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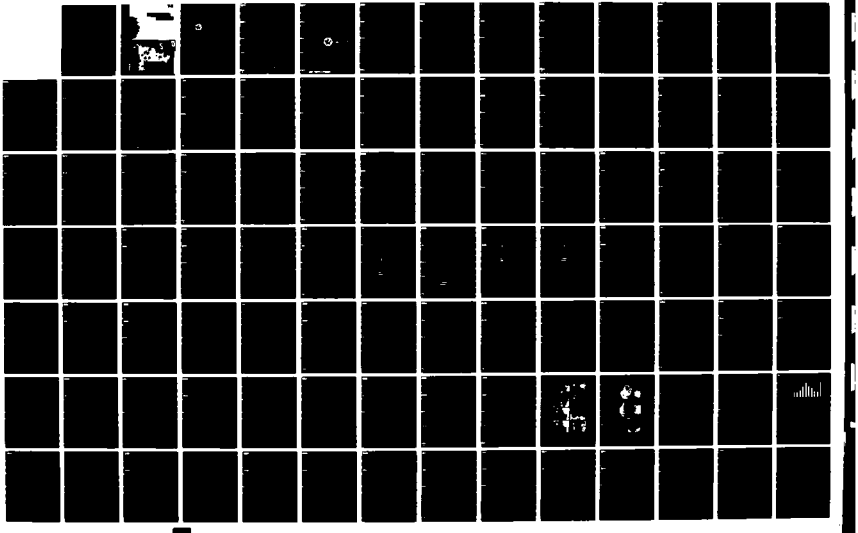

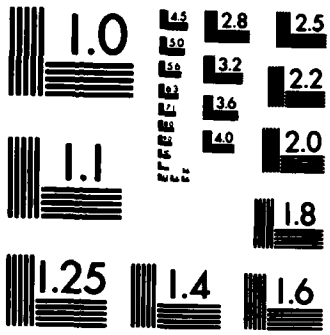

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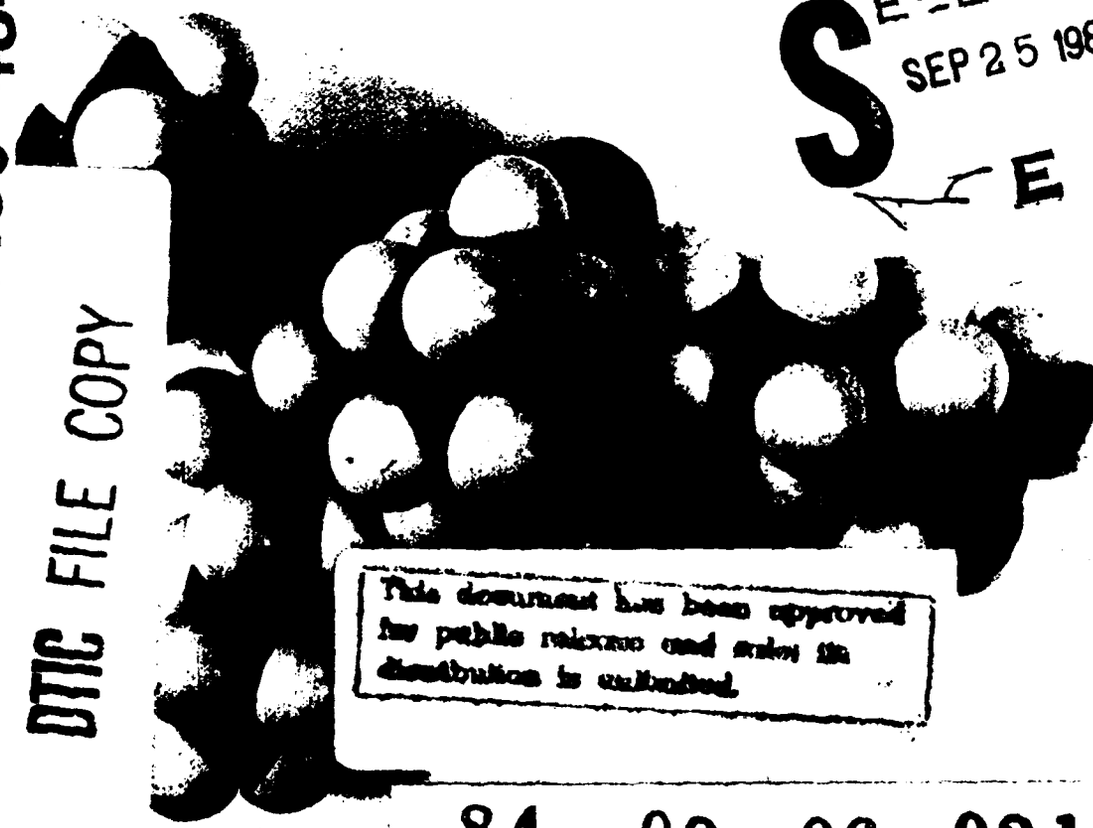

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ANTINEOPLASTIC, IMMUNOGENIC AND OTHER EFFECTS OF THE TETRAPEPTIDE TUFTSIN: A NATURAL MACROPHAGE ACTIVATOR

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ANTINEOPLASTIC, IMMUNOGENIC AND OTHER EFFECTS OF THE TETRAPEPTIDE TUFTSIN: A NATURAL MACROPHAGE ACTIVATOR[a]

Editors and Conference Organizers

VICTOR A. NAJJAR AND MATI FRIDKIN

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- UNITED STATES OFFICE OF NAVAL RESEARCH

PART I. RETROSPECTIVE

Tuftsin, A Natural Activator of Phagocyte Cells: An Overview[a]

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INTRODUCTION

Approximately three decades ago, my colleagues and I were able to show that upon interaction, antibody and antigen suffer a considerable change in conformation in the resulting complex such that new sites on both molecules unfold.[1-4] These sites, hitherto cryptic and shielded from the immunological system, are now exposed and subject to an immunogenic response. This results in the generation of specific antibodies that are directed not only towards the unfolded antigen sites but also towards the newly exposed areas on the antibody molecule. Thus auto-anti-antibody was born.[1-4] This implied, of course, that with every immunological challenge, the resulting antibodies would contain a population of auto-anti-antibodies that naturally augment the immune complex and if sufficiently large could and indeed do inhibit antibody activity.[5]

These findings, much appreciated in our laboratory, did not conform to the established notions of the day and did not escape the usual consequences.[4] In olden times, those who strayed and challenged established norms were swiftly banished, affixed to the cross, or callously stoned to death. It is comforting to learn that due credit for the original discovery of auto-anti-antibody was finally forthcoming.[6]

Auto-anti-antibodies, after a lapse of many years, were renamed anti-idiotypic antibodies and assigned a regulatory function in a network theory of the immune system.[7]

The proposal[1-5] and demonstration[8] of conformational change and consequent anti-antibody formation[3,4] led us to postulate the existence of a physiological role for γ-globulin.[3,4,9] This will not be discussed as it will require a lengthy exposition that may not be appropriate to this conference. This postulate soon proved to be correct.[3,4] My associates and I showed that specific erythrophilic γ-globulin binds to erythrocytes and is necessary for the survival of the red cell.[10,11] Similarly, platelets are coated with thrombophilic γ-globulin[12] that affects their respiration[13] and finally, leukophilic γ-globulin G (IgG) leukokinin binds only to blood granulocytes and stimulates their phagocytic activity.[14,15] This was later shown to be true of blood monocytes[16] and tissue macrophages.[17]

It was during a study of the kinetics of phagocyte stimulation that we made two relevant observations. The first was that the rate of phagocytosis by human or dog neutrophils did not proceed as expected when stimulated by the cytophilic leukokinin. It was our expectation at first that the mere binding of leukokinin to its receptor on the cell could trigger the necessary steps towards stimulation of the engulfing process. However, kinetic studies showed otherwise.[18,19] The addition of optimal concentrations

[a]This work was supported by the Public Health Service Grant 5RO1-A109116, the March of Dimes Birth Defects Foundation Grant 1-556 and by the American Cancer Society, Inc. Grant R.D.P. 32A.

of leukokinin to a reaction mixture of PMN and staphylococci did not result in a linear rate of phagocytic stimulation. On the contrary, after a short interval of time, the rate of stimulation fell off to control levels despite the fact that an excess of cells and target particles remained that did not participate in the reaction. However, when fresh leukokinin was added, stimulation was resumed only to fall off again after a similar lapse of time. Such anomalies were relegated to the difficulties inherent in the method used or to the vagaries of the cell. However, this observation reasserted itself time and again. It could not be further ignored. In essence, it suggested that somehow during the stimulatory reaction, leukokinin was being used up or spent in the process. (See FIG. 1.) The second observation came unexpectedly. During an experiment this author was performing, an interruption was imposed. The blood granulocytes and leukokinin were in place, mixed and awaiting the start of the reaction with the addition of the bacterial particles. However, the start of the reaction was postponed and over 30 minutes elapsed before the addition of the target bacteria. When this was finally done, no stimulation of phagocytosis occurred.[18]

This was the first time that leukokinin failed to stimulate the phagocytic cell. Again, the notion was reinforced that leukokinin was spent in a process that must have occurred during the long incubation before the addition of particles. In other words, by the time the particles were added, stimulation was over. In support of this was the fact that the addition of fresh leukokinin to the same cells resulted in the usual stimulation. This absolved the cells from any suspicion. They were still alive and well. By contrast, the recovered "spent leukokinin" was incapable of stimulating a fresh batch of cells. (See FIG. 2.) Soon thereafter, it became clear that a small fragment of leukokinin was taken up and processed by the cell. The leukokinin molecule did not suffer a measurable change in molecular weight. Furthermore, the activity of the fragment was equivalent to that of the parent leukokinin on a molar basis. It was destroyed by certain proteolytic enzymes.[18-20]

It was later found that an enzyme on the outer membrane of the granulocyte was responsible for the release of the activating fragment. It was named leukokininase. A preparation of this enzyme from the cell membrane of the granulocyte was later used to identify and isolate a tetrapeptide, threonyllysylprolylarginine (Thr-Lys-Pro-Arg), that was responsible for the full activity of the leukokinin molecule.[21] Similar preparations of macrophages from the peritoneal cavity and lungs of mice and guinea pigs were effective in the production of free tuftsin from leukokinin as measured by

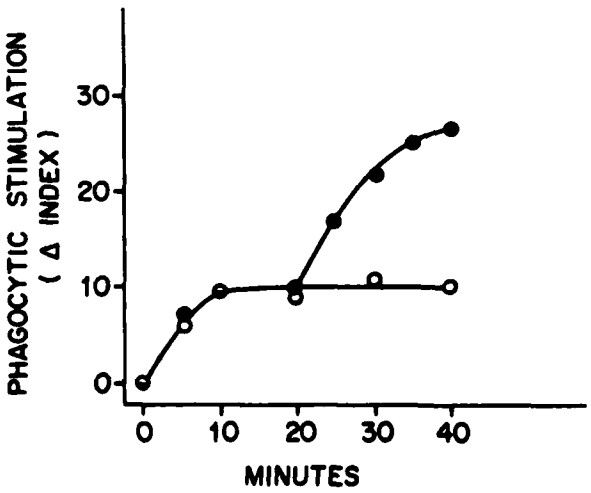

FIGURE 1. Short-lived stimulation of phagocytic activity by leukokinin. The reaction mixtures consisted of (O) 50 μg of fraction IV containing leukokinin, polymorphonuclear cells, and bacteria; (●) a similar reaction mixture, except that at 20 min another 50 μg of fraction IV was added. At the indicated times, samples were taken, and the phagocytic index was determined by visual counting. (For details see the text.) From Nishioka *et al.*[20]

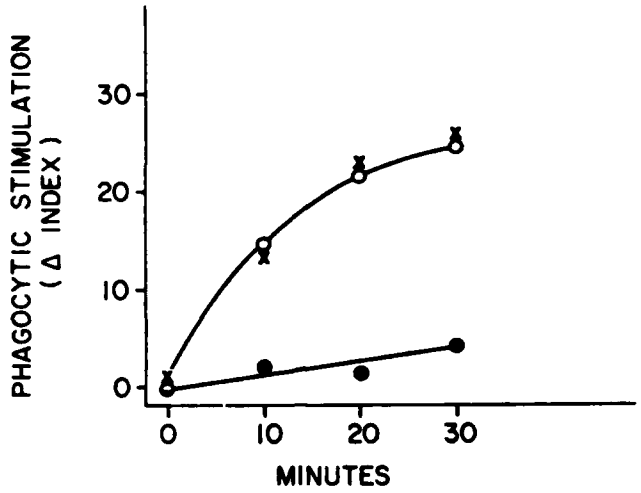

FIGURE 2. Loss of phagocytic stimulation by leukokinin after preincubation with thoroughly washed intact leukocytes. Under these conditions, intact erythrocytes show no effect on leukokinin activity. ●, 100 μg of fraction IV containing leukokinin, were preincubated with a human buffy coat preparation of polymorphonuclear cells for 20 min in Krebs-Ringer buffer, pH 7.4 at 37°C, and only bacteria were added at zero time; O, a similar preparation was made, except that bacteria with another 100 μg of fraction IV were added together at zero time; x, 100 μg of fraction IV, cells, and bacteria were added rapidly within 30 sec without preincubation. As indicated, samples were taken at 10, 20, and 30 min, and the phagocytic index was determined by visual counting. (For details see the text.) From Nishioka *et al.*[20]

phagocytic stimulation of protein-free tuftsin extracts. These macrophages were also stimulatable by tuftsin to the same extent as blood and peritoneal neutrophils.[17]

MATERIALS AND METHODS

The following were purchased: DBA/2 mice (Jackson Laboratories, Bar Harbor, ME), C57B1/6 (Charles River Laboratory, North Wilmington, MA), L1210 cells (American Type Culture Collection, Rockville, MD) amino acids (Bachem, Inc., Torrance, CA) protected with Boc at N^{α}, threonine as O-Bz, lysine as N^{ϵ}-Z, and arginine as N^{G}-tosyl. All coupling steps were carried out with dicyclohexylcarbodiimide (Pierce Chemical Co., Rockford, IL).

Specific leukophilic γ-globulin was purified by phosphocellulose chromatography.[14,15] Phagocytosis stimulation was performed on dog or human buffy coat with the classical method using *Staphylococcus aureus* as target particle.[14,15] Tuftsin was synthesized[19,20] or purchased from Sigma Chemical Co., (St. Louis, MO) and purified by high performance liquid chromatography using 0.075% trifluoroacetic acid and 3% methanol in water.

TUFTSIN, A TRUE BIOLOGICAL ENTITY

From all that has been done so far, it appears certain that tuftsin is a true biological entity with the principal function of activating phagocytic cells, principally the

macrophage. As with many biologically active peptides, it is a part of a parent carrier molecule leukokinin.[18 22] It is present in the CH2 domain of the Fc segment of the heavy chain, residues 289–292.[23] Two special enzymes serve to free it from the carrier molecule. A spleen enzyme, tuftsin-endocarboxypeptidase, nicks the heavy chain at the Arg-Glu bond between residues 292 and 293. The arginine carboxyterminal is now susceptible to the action of carboxypeptidase β. The leukokinin-S so nicked is present as such in the tissues and blood, free or bound to the outer membrane of the appropriate phagocyte. The membrane enzyme leukokininase acts on the bound leukokinin-S to cleave it at the amino end of threonine between residues 288 and 289,

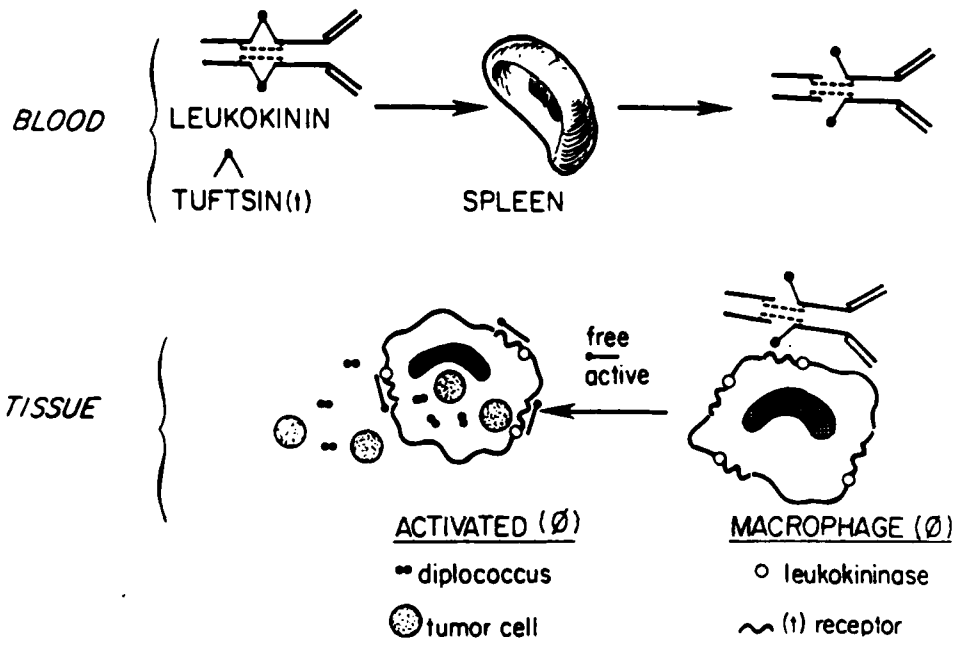

FIGURE 3. Leukokinin circulates through the spleen and is acted upon by tuftsin-endocarboxypeptidase, which cleaves the Arg-Glu bond at the carboxyterminal of tuftsin. The nicked molecule, still held together by disulfide bonds (-----), reenters the circulation. At this point, tuftsin is still covalently bound to this carrier leukokinin at its aminoterminal threonine. Finally, the carrier molecule binds to specific receptors on the outer membrane of a neutrophil, monocyte, or macrophage where tuftsin is released by the membrane enzyme leukokininase, which cleaves the Lys-Thr bond.

Tuftsin is fully active only as the tetrapeptide. If it is not set free from the parent carrier leukokinin, it remains inactive. Such a situation exists when the spleen is removed. In this case, the carrier leukokinin can still bind to the specific receptors at the cell membrane. Under these circumstances, tuftsin cannot be released. Consequently, it remains inactive and no stimulation of phagocytosis above basal levels can occur.

-Lys-Thr-. Tuftsin is now freed from the parent molecule and only as such is biologically active.[19,22,24-26] In this manner, the phagocytic cell plays a unique role in releasing its own activator. The sequence of events is illustrated in FIGURE 3.

Further proof that tuftsin is a biological entity is the existence of a mutation in the tetrapeptide in cases of congenital human tuftsin deficiency. We have studied 17 cases of tuftsin deficiency in the United States, 15 of which came from the Boston area. This attests to its possible high frequency.[26 29] Furthermore, two cases have been described in Japan.[30]

We have studied one patient extensively and identified the mutant peptide: Thr-Glu-Pro-Arg. Here one base change, GAA in place of AAA or GAG in place of AAG, results in the replacement of lysine by glutamic acid residue.[28,29]

It appears that the Fc portion of the heavy chain of γ-globulin G_1 (IgG_1) a carrier of the tetrapeptide tuftsin, is also a carrier of another peptide with biological activity. It is located further towards the carboxyterminal at residues 335–358 in the CH3 domain of EU IgG_1 sequence. It is responsible for the full activity of the Fc fragment in inducing murine B cells to secrete polyclonal antibody.[31]

It is my prediction that the highly versatile γ-globulin molecule will prove to be a carrier of several other physiologically active peptides that, like tuftsin and the peptide described above, will be located in the constant region. This gives substance to the question as to why nature intended to conserve the structure of the constant region of the heavy chain.

THE METABOLISM OF TUFTSIN

As indicated above, tuftsin is present in the CH2 domain of the Fc fragment of the heavy chain of the IgG_1, leukokinin, molecule. It circulates in the blood and is cleaved at the arginyl bond through the action of the spleen enzyme. This processed leukokinin is referred to as leukokinin-S.[22,25] Since the Fc structure is conserved, it is now known that the sequence of tuftsin, Thr-Lys-Pro-Arg, is present in all Fc segments of all four classes of IgG molecules at the same sequence location.[32] However, only leukokinin-S binds productively to the phagocytic cell and is the only IgG_1 molecule that harbors tuftsin with an arginine residue that has a free carboxyl group. As a consequence, it is susceptible to inactivation by the action of carboxypeptidase-β. What function tuftsin possesses in the rest of the IgG population other than leukokinin-S remains to be determined.

One of these possible functions was reported recently.[33] The results obtained suggest that the binding of antigen-human IgG complex to the first complement, C1, involves four residues, His-285, Lys-288, Lys-290, Arg-292, of the Cγ 2 region of the Fc portion. These four residues are in the sequence Val-Gln-Val-His-Asn-Ala-Lys-Thr-Lys-Pro-Arg, residues 282–292, interact with complementary sites of C1q to bind this subcomponent to human IgG.

It is clear from this that the tuftsin sequence encompasses the last four residues of this oligopeptide and presumably plays a significant role in this binding. In fact, it was shown that the tuftsin tripeptide inhibits 50% of C1-mediated immune hemolysis at a concentration of 750 μM.[33] This then assigns a new role for tuftsin in antibody function and immune hemolysis in addition to that furnished by leukokinin-S, which is the parent molecule designed to supply free and active tuftsin.

Tuftsin binds to its receptor after which it is internalized and becomes susceptible to the action of cytoplasmic enzymes.[34,35] Some of these represent novel enzymes. The most active enzyme is an aminopeptidase that cleaves off the threonine residue to yield the tripeptide Lys-Pro-Arg, which is an inhibitor[36–38] and regulator of tuftsin activity.[29,39,40] Finally, in the cytoplasm, tuftsin is fragmented to its constitutent amino acids.[35]

TUFTSIN AND SOME NATURAL ANALOGUES

In addition to its appearance in all four classes of IgG,[32] the tuftsin sequence occurs in guinea pig G_2 exactly at the same location, 289–292, as human γ-globulin.[41] Mouse

G_1 (MOPC 21), an analogue at the sequence Thr-Gln-Pro-Arg[32] appears at the same location and represents one base change at the first base of the triplet code.

Other than in the conserved areas of the CH2 region of immunoglobulin, a tuftsin sequence would be expected once in 20^4 or 1:160,000. Consequently, one should be alert to the possibility that if it does occur, it is likely to have some functional significance. In one particular instance, this might possibly be the case. Tuftsin sequence appears in residues 9–12 from the amino terminal of the P12 protein of Rauscher murine leukemia virus.[42] Treatment of cells infected with this virus resulted in a threefold increase in virus-associated reverse transcriptase.[43] Similar results were obtained with Kirsten Sarcoma virus grown on mouse cells. Tuftsin at 0.01–100 μg/ml induces endogenous virus within 3–4 hr. It appears that RNA synthesis is necessary since actinomycin D blocked the process.[44]

Thr-Arg-Pro-Lys is another natural tetrapeptide, where Lys and Arg exchange places in the tuftsin sequence. It is found at residues 214–217 in yet another virus protein; the influenza hemagglutinin JAP HA (H2, "57).[45] A role in hemagglutination is an attractive possibility because of its highly positive charge that would bind to membrane acidic groups. Canine tuftsin has been shown to be Thr-Lys-Pro-Lys, but its location in the active heavy chain has not been determined.[35] There is another analogue, Thr-Arg-Pro-Arg, in which the arginine residue replaces lysine in the tuftsin sequence. This analogue appears in the biologically active pancreatic polypeptide at residues 32–35 at the extreme carboxyterminal end next to the terminal residue tyrosinamide.[46] The pancreative polypeptide is presumed to have gastrointestinal functions. It is secreted after a protein meal and leads to a decrease in weight of certain genetically diabetic mice.[47] This tetrapeptide is present in all analogous pancreatic peptides of human, bovine, ovine, porcine, and canine origin. It must be emphasized that Thr-Arg-Pro-Arg, Thr-Lys-Pro-Lys, and Thr-Arg-Pro-Lys are about as active as tuftsin Thr-Lys-Pro-Arg.[48-54]

ACTIVATION OF PHAGOCYTIC CELLS, MACROPHAGES, AND GRANULOCYTES

Stimulation of Phagocytosis

Tuftsin was discovered[21] and identified[18,20] through the use of the classical phagocytosis assay. Crude as this assay is, it was sufficient for that purpose.[36-38,48-51] The K_m for phagocytosis was found to be 100 nM.[17] However, a more quantitative radioimmunoassay was developed by Fridkin's group[37] and reproduced in our laboratory.[39] In both instances arginine was the tritium-labeled residue because only arginine was available in sufficiently high specific activity for the assay. The stimulation of phagocytosis had been documented in several laboratories. At the Weizmann Institute, with the usual phagocytosis assay, the same K_m was obtained.[38] Several other groups utilized phagocytosis as the biological system to assess the activity of tuftsin and its analogues. Among these are Konopinska *et al.*,[52-54] Martinez *et al.*,[55] Vicar *et al.*,[56] Inada *et al.*,[30] Yajima *et al.*,[57] and Hisatsune *et al.*[49] Although tuftsin stimulates pinocytosis by phagocytic cells, it does not stimulate pinocytosis by cultured cell line mouse leukemia L1210, 3T12, and 3T6 cells.[58]

It must be stressed that all inhibitor analogues of tuftsin inhibit only tuftsin stimulation of phagocytosis. They do not inhibit the basal phagocytic activity of the cell. This may indeed mean that the basal and the stimulated activities are governed by independent mechanisms.

Stimulation of Motility

Nishioka *et al.*[20] showed that motility of neutrophils is stimulated by tuftsin in capillary tubes. No stimulation was observed with the inhibitor [Pro-Pro^3]-tuftsin. On the contrary, it inhibited the stimulation by tuftsin.

Activation of Immunogenic Functions

The activation of the immunogenic functions was first shown by Tzehoval *et al.*[59] The augmentation of this function in the presence of tuftsin was over 700%. Subsequently, Florentin *et al.*[60] gave direct evidence for the stimulation of antibody formation following tuftsin injection into mice at 20 mg/kg. There was an increase in antibody-forming cells in the spleen of more than threefold over control mice not injected with tuftsin.

Stimulation of the Bactericidal Activity

This was demonstrated in mice by Martinez *et al.*[55] who showed accelerated killing and clearing of several types of bacteria from the blood of mice treated with tuftsin (10–20 mg/kg).

Stimulation of the Tumoricidal Activity of the Macrophage

The most interesting *in vivo* observation has now been reported by several groups in this country and abroad. The growth in mice of several types of syngeneic tumor cells has been shown to be suppressed or eliminated by treatment with tuftsin. These comprise several types of melanoma including Cloudman S-91,[61] B16/5B,[62] L1210 leukemia cells,[39,60,61] fibrosarcoma,[63,64] and several lymphomas and adenocarcinomas.[65]

SUMMARY

The recognition that a small oligopeptide was responsible for the full stimulation effect of specific cytophilic γ-globulin on blood neutrophils arose from a study of the kinetics of phagocytosis. These were unusual in that the stimulation was short lived and that preincubation of the phagocyte with the γ-globulin rendered the latter inactive. The oligopeptide was isolated, its structure determined (Thr-Lys-Pro-Arg) and synthesized. The discovery of human mutants with tuftsin deficiency exhibiting signs and symptoms of frequent severe infection further emphasized the specific biological function of the tetrapeptide. The mutant peptide was isolated, sequenced (Thr-Glu-Pro-Arg), and synthesized. Further studies showed that tuftsin requires two enzymes for its liberation from the parent carrier γ-globulin. One enzyme is in the spleen that cleaves distal to the arginine end, and the other, on the outer side of the plasma membrane, cleaves proximal to the threonine residue.

The tetrapeptide tuftsin stimulates all functions of phagocytic cells: phagocytosis, pinocytosis, motility, immunogenic activity including processing of the antigen and augmentation of the number of antibody-forming cells, bactericidal activity, and, above all, tumoricidal activity. The latter has been shown by several laboratories.

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This paper is dedicated to Professor Sam M. Rapoport of Humboldt Universität zu Berlin, Institut für Physiologische und Biologische Chemie, Berlin, on the occasion of his 70th birthday.

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Synthetic Pathways to Tuftsin and Radioimmunoassay

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INTRODUCTION

Ever since the isolation and structural identification of the phagocytosis-stimulating peptide tuftsin as the basic tetrapeptide L-Thr-L-Lys-L-Pro-L-Arg[c] (Najjar and colleagues[1,2]), it has been chemically synthesized in many laboratories, using a variety of synthetic pathways (for review, see refs. 3, 4). The first synthesis of tuftsin, most naturally, was achieved by Najjar and coworkers who employed the solid-phase methodology.[2] Various other synthetic routes reported included conventional preparation in homogeneous liquid phase and are based on the employment of different condensing agents and strategies of peptide chain assembly as well as the use of a variety of side-chain protecting groups (e.g., refs. 5–12), or solid-phase techniques.[2,13] We have reported on three synthetic pathways to tuftsin based on either the polymeric-reagent approach,[8] or two different strategies in solution.[8,12] All of the synthetic models described gave tuftsin preparations that were identical in their biological activity in various assay systems,[3] as well as their physicochemical characteristics.

Recent studies have strongly hinted at the promising clinical potential of tuftsin as an immunostimulating, antibacterial, and antitumor drug (for review, see refs. 3, 14–16). In view of this fact and in order to satisfy the increasing demands for large amounts of tuftsin needed for toxicological studies in animals, and for initial clinical tests in humans, we have reevaluated our earlier synthetic work and explored new preparative routes to tuftsin.

Our studies were aimed at the achievement of the following criteria: (1) simplicity—use of convenient condensing methods and choice of protecting groups that can, preferably, be simultaneously and mildly removed at the end of synthesis; (2) efficiency—high yield and good reproducibility; (3) possibility for a facilitated scaling-up of synthesis; and (4) maximal purity of final product through avoidance of

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[c]Abbreviations for amino-acid derivatives and peptides follow the IUPAC-IUB Commission of Biochemical Nomenclature Symbols, see *Eur. J. Biochem.* **27**: 201–207 (1972).

potential inhibitors. Our earlier, as well as recent, synthetic work is discussed in the following sections.

SYNTHESIS USING POLYMERIC REAGENTS

Synthesis of tuftsin using the polymeric reagents strategy is depicted in FIGURE 1. The principle of such synthesis is the elongation of a soluble peptide chain through its amino terminal with the aid of insoluble active esters.[17] As excesses of such reagents can be employed and be easily removed at the end of synthesis from the reaction mixture, coupling yields are, as a rule, nearly quantitative. Our earlier synthesis of tuftsin, using insoluble active esters derived from (4-hydroxy-3-nitro)benzylated

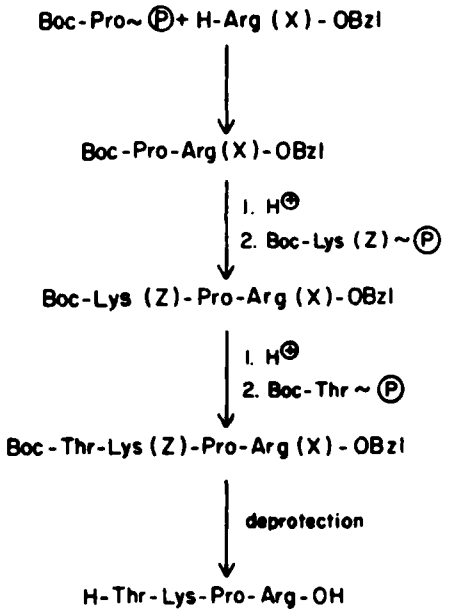


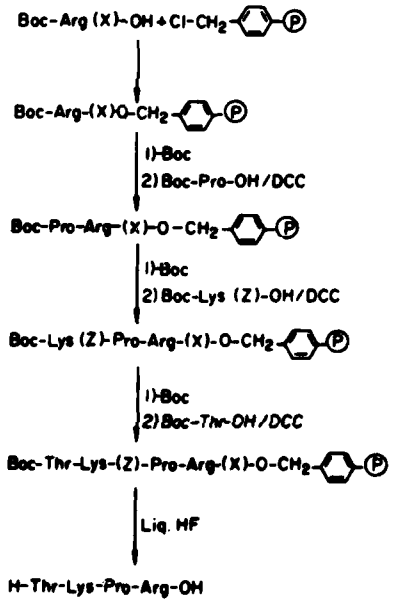
FIGURE 1. Synthesis of tuftsin using the polymeric-reagents approach.

polystyrene (PHNB), as reagents, was most efficient and gave protected tuftsin, Boc-Thr-Lys(Z)-Pro-Arg(NO_2)-OBzl, in an overall yield of 92% and free tuftsin in yields higher than 80%.[8] Scaling up of this synthesis led, however, to two major drawbacks: first, reduced coupling times; second, substantial side reactions during the final synthetic step, primarily at the NO_2-protected guanidine side chain of the Arg-4 residue, that is, the arginine → ornithine transformation. Both problems could be solved when using as reagents, active esters derived from polystyrene-bound 1-hydroxybenzotriazole (PHBT), and the tosyl (Tos) or the mesitylene sulfonyl (Mts) groups for protection of arginine (FIG. 1). Intermediate peptides were obtained in high yields and purity without the necessity of crystallization. Pure tuftsin was obtained

after fractionation of the crude product by ion exchange chromatography on a Dowex AG 50W-X4 column, in high yields (>80%). Synthetic manipulations, however, with large amounts of PHBT (~50 g) are not yet convenient and routine. As continuous, mechanized, columnwise or batchwise synthesis of peptide using the polymeric reagents approach is feasible and advantageous,[17] we are still pursuing tuftsin synthesis along this route.

SOLID-PHASE SYNTHESIS

Preparation of tuftsin employing the solid-phase methodology is perhaps the most straightforward synthetic route. Najjar and his colleagues adopted this attractive technology for their syntheses, which yielded pure and biologically active peptide.[2,13] Our studies on the solid-supported synthesis of tuftsin are illustrated in FIGURE 2. We have employed two types of carriers: (a) commercially available bead-type polystyrene cross-linked with either 1% or 2% divinylbenzene, and (b) commercially available, bead-type macroporous polystyrene. The *tert*-butyloxycarbonyl (*t*-Boc) group was used for α-amino protection, the ϵ-amino function of lysine was masked by the benzyloxycarbonyl (Z) group, the guanidine side chain of arginine was protected by the nitro (NO_2), tosyl (Tos), or mesitylene sulfonyl (Mts) group while the hydroxy function of threonine was left unprotected. Syntheses usually proceeded smoothly, yielding tuftsin, after purification of crude products primarily by ion-exchange



Ⓟ-⬡-CH_2-Cl : Chloromethyl polystyrene

X = NO_2, Tos, Mts ; Z = benzyloxycarbonyl

FIGURE 2. Synthesis of tuftsin using the solid-phase approach. In the procedure described by Najjar and colleagues,[2] the following amino acid derivatives were used: Boc-Arg(NO_2)-OH, Boc-Pro-OH, Boc-Lys(Z)-OH and Boc-Thr(Bzl)-OH.

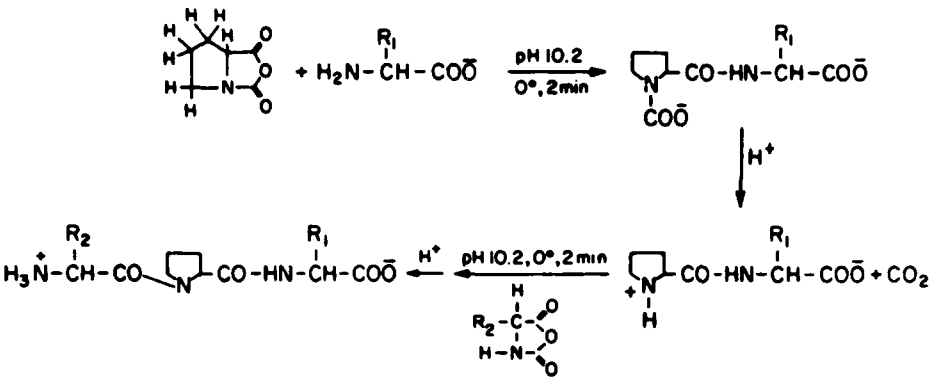


FIGURE 3. General Scheme for the synthesis of H-Lys(Z)-Pro-Arg-OH using the N-carboxyanhydride approach. R_1 = -$(CH_2)_3$-NH-C(=NH)-NH_2; R_2 = -$(CH_2)_4$-NH-CO-O-$CH_2C_6H_5$.

chromatography, in good yields (30–40%). Examination of the synthesis reveals, however, that the large excesses of solvents and of the Boc-amino-acid derivatives (3–4 equivalents) employed in each coupling step, turn the preparation of this short peptide using the solid-phase approach economically impractical. Moreover, handling of large volumes of anhydrous HF (in large-scale preparations), was found, in our hands, rather tedious. This may give rise, as we will see later, to breakdown products and some side reactions.

SYNTHESIS USING N-CARBOXYANHYDRIDES

Assembly of a peptide chain using the N-carboxyanhydride (NCA) method, is most attractive since it is a fast, high-yield procedure that needs relatively simple and inexpensive starting materials, and above all, does not require NH_2-terminal protection.[18] These beneficial features were used in the synthesis, by the Merck group, of the S protein of ribonuclease A.[19] Attempting however, to apply the NCA approach to the synthesis of tuftsin (FIG. 3), we observed the extensive formation of side products, mostly elongated peptides. In fact, we were not able to proceed, in reasonable yields, beyond the stage of H-Lys(Z)-Pro-Arg-OH. This is primarily due to the strict reaction conditions (see FIG. 3), which require expertise and much training for the successful execution of the reaction. Synthesis using N-carboxyanhydrides can also be accomplished in a heterogeneous mixture of acetonitrile and water (~1:1, v/v), at a subzero temperature (~ −10°C) in the presence of Na_2CO_3 (pH ~ 10).[20] Peptide bond formation occurs, supposedly, at the interphase area. Using these less strict reaction conditions, we have synthesized tuftsin employing NCA-derivatives throughout the preparation (FIG. 4) or in combination with N-hydroxysuccinimide active esters (FIG. 5). Reproducibility of the first procedure was rather poor, mostly due to the high sensitivity of NCA-Pro to self polymerization. Synthesis using the later procedure (FIG. 5) afforded pure tuftsin (at a 5-mmol scale) in 32% overall yield. Scaled-up experiments (~50 mmol) exposed some technological problems such as the difficulty in preservation of constant cooling, or of the efficient and uniform mixing of phases. Due to these difficulties, precipitation of starting materials as well as products, often occurred and led to incomplete reactions and a variety of side products. The use of

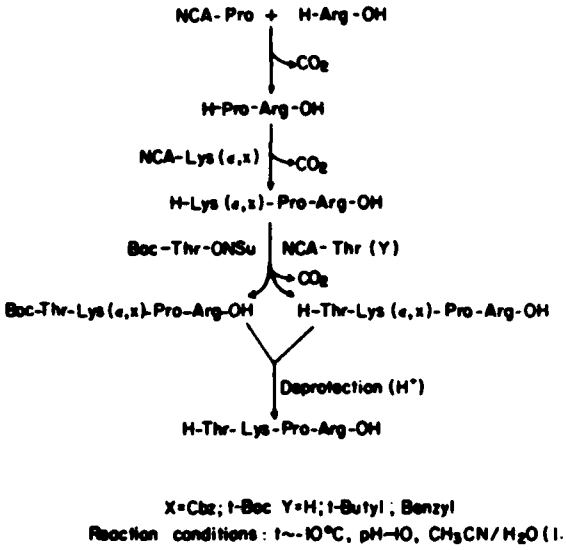


FIGURE 4. Synthesis of tuftsin using the N-carboxyanhydride procedure in a heterogeneous reaction mixture.

large amounts of acetonitrile, a notoriously toxic solvent, may also create operational obstacles.

CONVENTIONAL SYNTHESIS IN SOLUTION

Two "conventional" routes to tuftsin were reported by our laboratory. The first[8] (FIG. 6), led to the protected tuftsin derivative Boc-Thr-Lys(Z)-Pro-Arg(NO_2)-OBzl (similar to that obtained in the polymeric reagents route), or alternatively, to Z-Thr-Lyz(Z)-Pro-Arg(NO_2)-OBzl, both obtained in about 20–30% overall yield. Upon scaling up the reaction from ~2 mmoles to 25 mmoles, several side products were encountered. These were generated primarily during the fina! deprotection step of harsh acidolysis with anhydrous HF, some of which involved the arginine residue. Similarly, deprotection using a combination of milder acidolysis (HBr-CF_3COOH) with catalytic reduction (H_2-Pd/$BaSO_4$) was often incomplete and yielded side

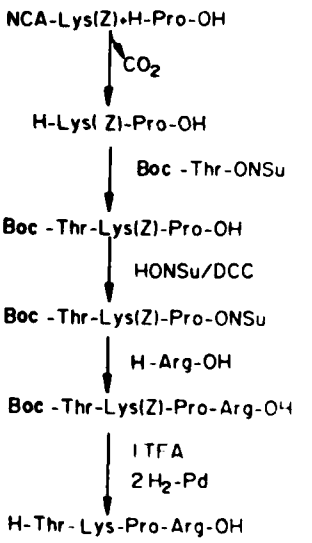


FIGURE 5. Synthesis of tuftsin by a combined heterogeneous N-carboxyanhydride method and N-hydroxysuccinimide ester derivatives.

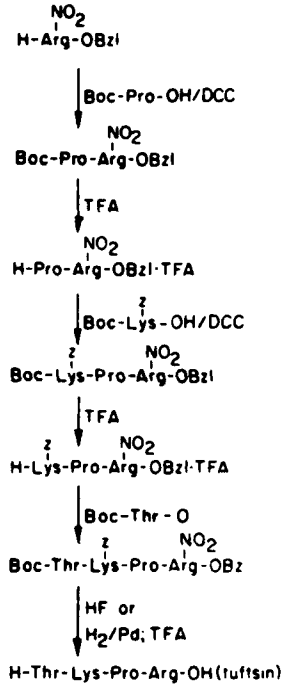


FIGURE 6. Synthesis of tuftsin in solution.

products. The final synthetic step thus brought about a considerable decrease in yields of pure tuftsin. Consequently, an alternative pathway was developed,[12] in which the arginine residue is introduced into the peptide chain as a free amino acid. The synthetic route is summarized in FIGURE 7. The overall yield of pure tuftsin in the later procedure was about 25%. The various synthetic steps were reproducible when synthesis was carried out at a 5–10-mmol scale. The basic hydrolysis, however, of Boc-Thr-Lys-(Z)-Pro-OBzl to afford Boc-Thr-Lys(Z)-Pro-OH, gave rise to increasing amounts (up to 30%) of two unidentified side products (FIG. 8). This was particularly

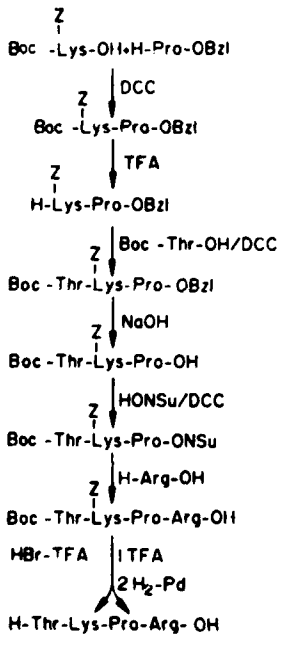


FIGURE 7. Synthesis of tuftsin in solution involving an unprotected arginine residue.

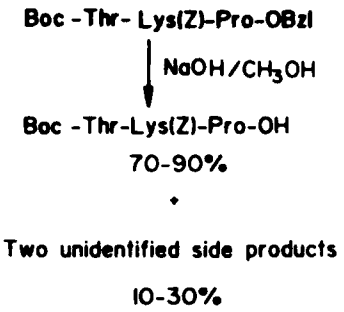


FIGURE 8. Basic hydrolysis of Boc-Thr-Lys(Z)-Pro-OBzl.

evident at large synthetic scales. Removal of contamination from the desired product could be achieved by selective precipitations but at the expense of yields. Final deprotection of the blocked tuftsin derivative could be easily achieved (see FIG. 7) using a combination of acidolysis by trifluoroacetic acid followed by catalytic reduction (H_2-Pd/C) or by treatment with HBr in CF_3COOH or with anhydrous HF. The former procedure of deprotection was a preferable one since it yielded rather pure free tuftsin. The latter two acidolytic procedures, on the other hand, gave several side products (FIG. 9). Noteworthy is a putative cyclic product composed of equimolar amounts of proline and arginine, has no amino terminal, gives a positive Sakaguchi test for guanidine and is different in its physicochemical properties from an authentic sample of the diketopiperazine of Pro-Arg. This product, most probably a tuftsin inhibitor, is eluted from a Dowex-50 column close to tuftsin. Repetition of ion-exchange chromotography is sometimes required for its complete removal.

Another side reaction worth mentioning may occur under conditions of basic hydrolysis, especially when employing large excesses of base and prolonged reaction time. This is a ring closure at N-benzyloxycarbonylthreonyl-residues, leading to the corresponding 2-oxo-oxazolidine ring compounds (FIG. 10).[12]

Upon examination of the various synthetic routes to tuftsin, we designed a pathway that encompasses the answers to the above-mentioned problems. The procedure, which does not include basic hydrolysis nor harsh acidolysis, is summarized in FIGURE 11. Basically, the procedure is similar to the previous one (FIG. 7), the only difference being the protection of the side-chain of lysine by the *tert*-butyloxycarbonyl group instead of the benzyloxycarbonyl and similar substitution at the α-amino position. The overall yield of tuftsin, in synthetic scales of up to 20 mmols, is about 30%, preparations are reproducible, protecting groups removed mildly and simultaneously, side-product formation is minimal, and attainment of pure tuftsin, through ion exchange chromatography, is rather simple and convenient.

It seems to us that the last-described pathway for synthesis of tuftsin fulfills the

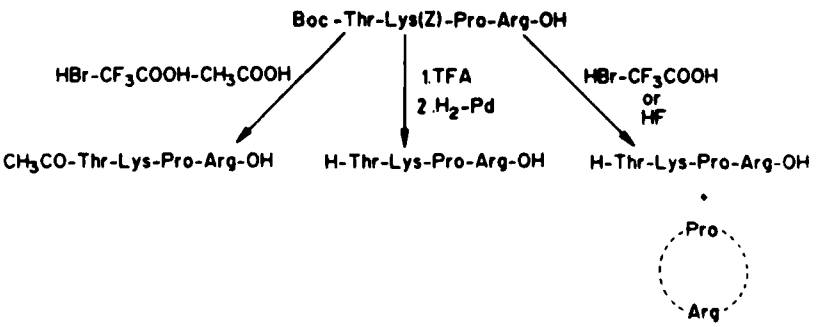


FIGURE 9. Side products obtained by acidolysis of protected tuftsin.

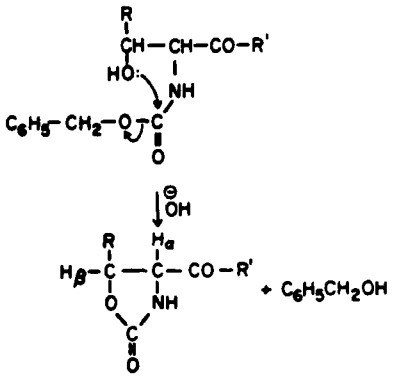


FIGURE 10. Cyclization of N-benzyloxycarbonyl derivatives of threonine under alkaline conditions. R=CH_3; R'=OMe, OEt or -NH-peptide.

four criteria stated in the introduction. We are currently adopting this procedure for the large-scale synthesis of the peptide.

CRITERIA FOR PURITY OF TUFTSIN

It was reported by several investigators that synthetic preparations of tuftsin may contain potent peptidic inhibitors.[3,13,14] These derivatives, some of which closely resemble tuftsin in their physicochemical features, may hamper, both *in vivo* and *in vitro,* biological functions of tuftsin. Before every biological study, the purity of our synthetic preparations is routinely and carefully checked and has to fulfill the following criteria:

(1) Homogeneity, ninhydrin and Sakaguchi positive spot, on high-voltage paper electrophoresis—Whatman No. 3 paper, 45 min at 60 V/cm in pyridine-acetate buffer (pH 3.5);

(2) Homogeneity on HPLC, using C-18 column, employing a solution of 0.3% isopropyl alcohol in aqueous 50 m*M* NH_4Ac, pH 6.5, as eluent;

(3) Correct amino acid ratio after exhaustive acid hydrolysis—6 *N* HCl containing 4% phenol at 110°C for 20 hr, in evacuated sealed tubes;

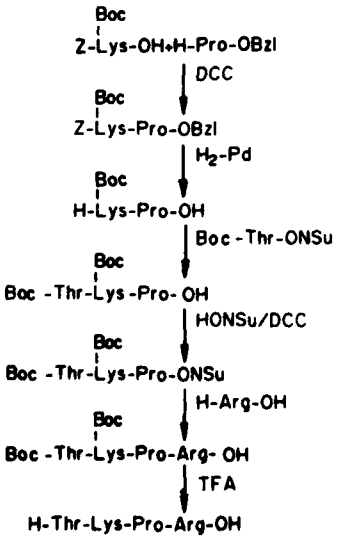


FIGURE 11. Synthesis of tuftsin in solution involving an unprotected arginine residue.

(4) Capacity to compete with [^{3}H-Arg^4]tuftsin on binding to specific receptor sites on macrophages, using a standardized assay.[21]

RADIOIMMUNOASSAY OF TUFTSIN

In order to render synthetic tuftsin antigenic, *p*-diazonium phenylacteyltuftsin was coupled to various proteins: ribonuclease A, ovalbumin, Keyhole limpet hemocyanin (KLH), tyroglobulin and bovine serum albumin (BSA). It was also coupled to a multichain synthetic copolymer of L-lysine, L-tyrosine and DL-alanine (poly-DL-Ala-poly-L-Tyr-poly-L-Lys, mol. wt. = 142.500). Coupling proceeded smoothly at pH 8.5 for 10 hr at 4°C, and the extent of loading of the high-molecular-weight carrier with tuftsin moieties could be easily manipulated by varying the hapten/protein molar ratio. Extent of protein loading can be determined by amino-acid analysis of the product conjugate. Using the tuftsin-carrier conjugate to immunize rabbits, antisera were obtained that bound ^{125}I-labeled *p*-aminophenylacetyl-tuftsin. The BSA-tuftsin conjugate was by far the most antigenic when each protein molecule carried 20–40 tuftsin residues (FIG. 12). Antisera obtained in this manner were capable of binding the ^{125}I-tuftsin derivative at dilutions up to 1:1500 (maximal binding 25–30%).[22] Binding of the radiolabeled peptide was inhibited by tuftsin and some of its synthetic NH_2-terminal analogues but was not affected by various unrelated natural and synthetic peptides (FIG. 13). The amino-acid sequence Lys-Pro-Arg-OH appears to be the antigenic determinant recognized by the rabbit antibodies.

Attempting to prepare antibodies that recognize the whole tuftsin molecule, we have recently prepared BSA-tuftin conjugates in which the tuftsin residue is attached to the protein carrier at its COOH-terminus. This was achieved in the manner shown in

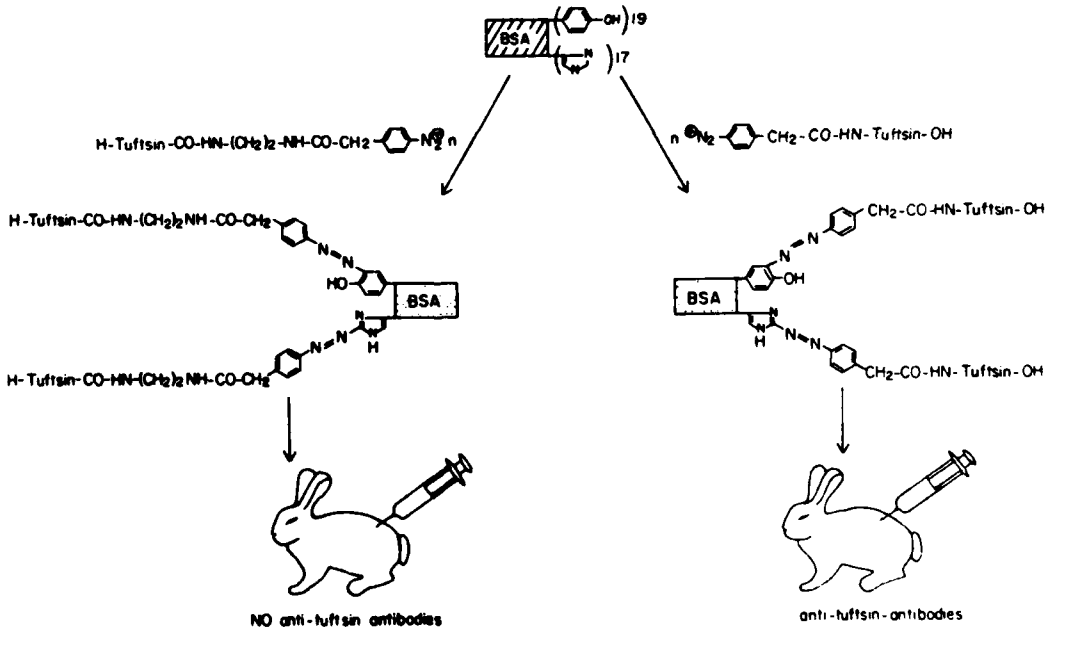


FIGURE 12. Preparation of BSA-tuftsin conjugates and of antituftsin antibodies.

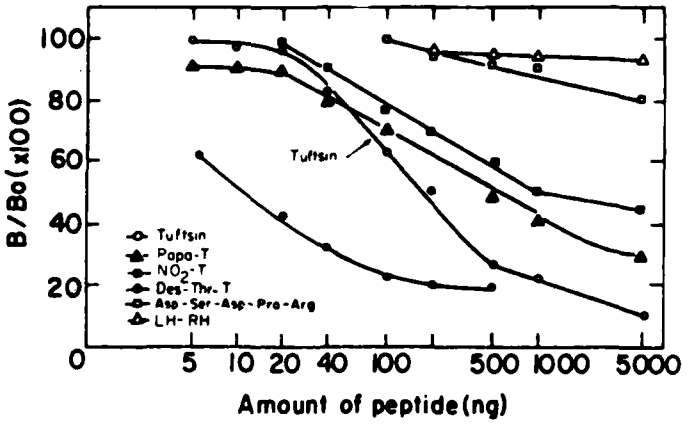


FIGURE 13. Inhibition of binding of ^{125}I-labeled *p*-aminophenylacetyl-tuftsin to antiserum by tuftsin, tuftsin analogues, and unrelated peptides.

FIGURE 12. Immunization of rabbits with this conjugate produced antibodies that are capable of binding to the hapten, that is, H-Tuftsin-CO-NH-$(CH_2)_2$-NH-CO-CH_2-C_6H_4-NH_2-*p*, but not to tuftsin (FIG. 12). The finding is rather surprising, especially in view of the fact that *p*-aminophenylacetyl-tuftsin (an NH_2-terminal analogue) is biologically inactive and is rather poorly capable of competing with [^{3}H-Arg^4]tuftsin on binding to receptor sites on macrophages. The COOH-terminal derivative of tuftsin, on the other hand, is biologically active and can bind to tuftsin receptors.

Examination of the levels of tuftsin in human blood, using the radioimmunoassay technique, reveal that only negligible amounts are present as such in circulation. Upon partial trypsinization of serum, the amounts of tuftsin or of immunochemical material related to tuftsin are markedly increased, indicating that the peptide circulates in the body while primarily attached to its carrier protein, leukokinin.

Using the radioimmunoassay method, quantitative determination of tuftsin, or of tuftsin-related peptides, was performed in the trypsin-treated sera of normal and splenectomized humans, as well as of patients suffering from various splenic and immunologic malfunctions. Lower levels of tuftsin were generally detected in subjects who underwent elective splenectomy or who suffer from impaired splenic-function.[3,22-24] It is not our intention to discuss here these results and their clinical implications. This will be described in detail in the lecture of Dr. Z. Spirer on acquired tuftsin deficiency in this symposium.

ACKNOWLEDGMENT

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New Synthetic and Natural Tuftsin-related Compounds and Evaluation of Their Phagocytosis-stimulating Activity

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INTRODUCTION

One of the defense mechanisms available to the host in its defense against invading microorganisms is phagocytosis, which plays a significant role in the critical early stages of bacterial infections, and thereby is an important determinant of the eventual outcome of these infections. Effective phagocytosis activity early in the course of bacterial invasion may limit the spread of bacteria and prevent continuing infection, while ineffective phagocytosis may lead to uncontrolled bacterial multiplication and overwhelming infection.[1]

Tuftsin, a tetrapeptide released from the Fc portion of the heavy chain of the IgG by specific enzymes[2] and isolated by Najjar and coworkers,[3-5] was shown to stimulate phagocytosis of PMN and macrophages.[3-6] In fact, it was found that tuftsin stimulates all known activities of the macrophage and granulocyte: motility,[7,8] hexose monophosphate shunt,[9] chemotactic effect,[10] MuLV production,[11,12] immunogenic function,[13-15] anti-infectious effect,[16,17] and tumoricidal activity,[14,15,18,19] as well as some analgesic effects were demonstrated for this compound.[20]

EARLIER STUDIES

It was shown in several laboratories that tuftsin stimulates phagocytosis by phagocytes. We found that *in vitro*, tuftsin stimulates phagocytosis by macrophages against *Staphylococcus aureus, Staphylococcus londres, Staphylococcus aureus Smith*, and *Listeria monocytogenes*, this stimulation being concentration dependent and efficient for concentrations of 5 and 10μg/ml of tuftsin. The action of tuftsin on intracellular bactericidal activity of macrophages was clearly established with *Listeria monocytogenes*. We have shown that stimulation of bactericidal activity of macrophages by tuftsin is greatly accelerated during the initial phase (15 minutes) of infection. Probably this stimulatory effect does not last longer than that time interval because of destruction of tuftsin by cell enzymes. Bactericidal activity of macrophages from the peritoneal cavity of mice injected intravenously with bacteria is enhanced by tuftsin and is increased with time. Injection of 10 or 20 mg of tuftsin per kilogram of body weight in normal mice raised bactericidal activity of macrophages up to 50% and 70%, respectively, during the first 15 min of infection, while bactericidal activity of the control yielded 5% of killing of bacteria. The same experiment in leukemic mice,

showed fairly good bactericidal activity as compared to tuftsin-untreated controls, but without reaching the level obtained in normal mice.

Because of the principal role of both liver and spleen in fighting bacterial infections, it appeared interesting to determine what happened to bacteria inside these organs in healthy and leukemic tuftsin-treated mice as compared to normal mice.

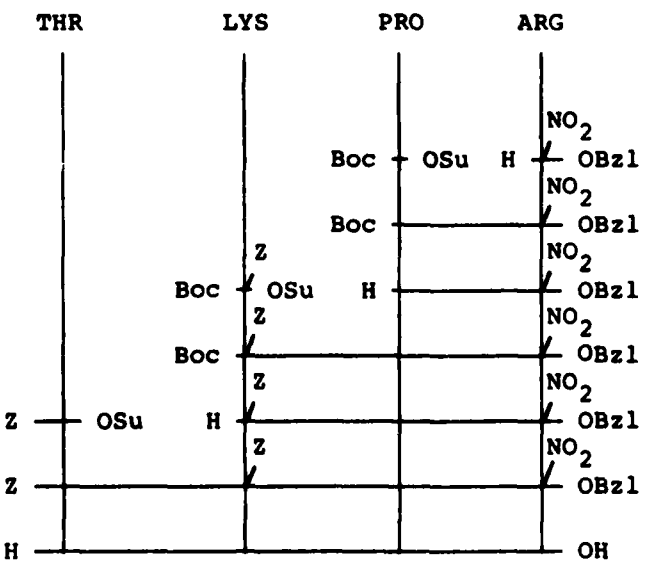
In fact, we showed that tuftsin stimulates bactericidal activity in the liver and in the spleen of tuftsin-treated animals at 10 or 20 mg/kg of body weight. However, leukemic animals did not show significant bactericidal activity in the liver as compared to some positive results obtained in the spleen.

Some beneficial effects were obtained on the rate of blood clearing in normal and leukemic mice infected by bacteria. It was quite obvious that rate of clearing upon injection of 10 or 20 mg of tuftsin per kilo of body weight in normal mice was noticeably greater than the control (without tuftsin) with the tested bacteria (*Staphylococcus aureus, Listeria monocytogenes, Escherichia coli,* and *Serratia marcescens*). Blood clearing of bacteria in leukemic mice showed that if tuftsin administration definitively stimulated bacterial clearing, the rate of clearing still did not reach the one obtained in normal mice.

Involvement in a major program with I. Florentin and G. Mathé concerning further studies of immunogenic and tumoricidal properties of tuftsin, made the need for significant amounts of this peptide obvious. On the other hand, it was shown that a high purity of tuftsin was necessary to obtain full biological activity.[21] In fact, impurities containing tuftsin were found to have inhibitory effects on phagocytic activity. According to K. Nishioka, thorough toxicological studies are essential before tuftsin can be submitted for approval of clinical trials and large-scale production of tuftsin is the key problem.[22] For this reason, we undertook the preparation of significant amounts of pure tuftsin.

SYNTHESIS OF TUFTSIN—PURIFICATION AND EVALUATION OF A METHOD ALLOWING ITS INDUSTRIAL DEVELOPMENT

We tested several methods of synthesis of tuftsin according to the literature and also by trying to improve protection and coupling methods. Among them, we selected one method of synthesis that gave us the best results.



SCHEME 1. Synthesis of tuftsin.

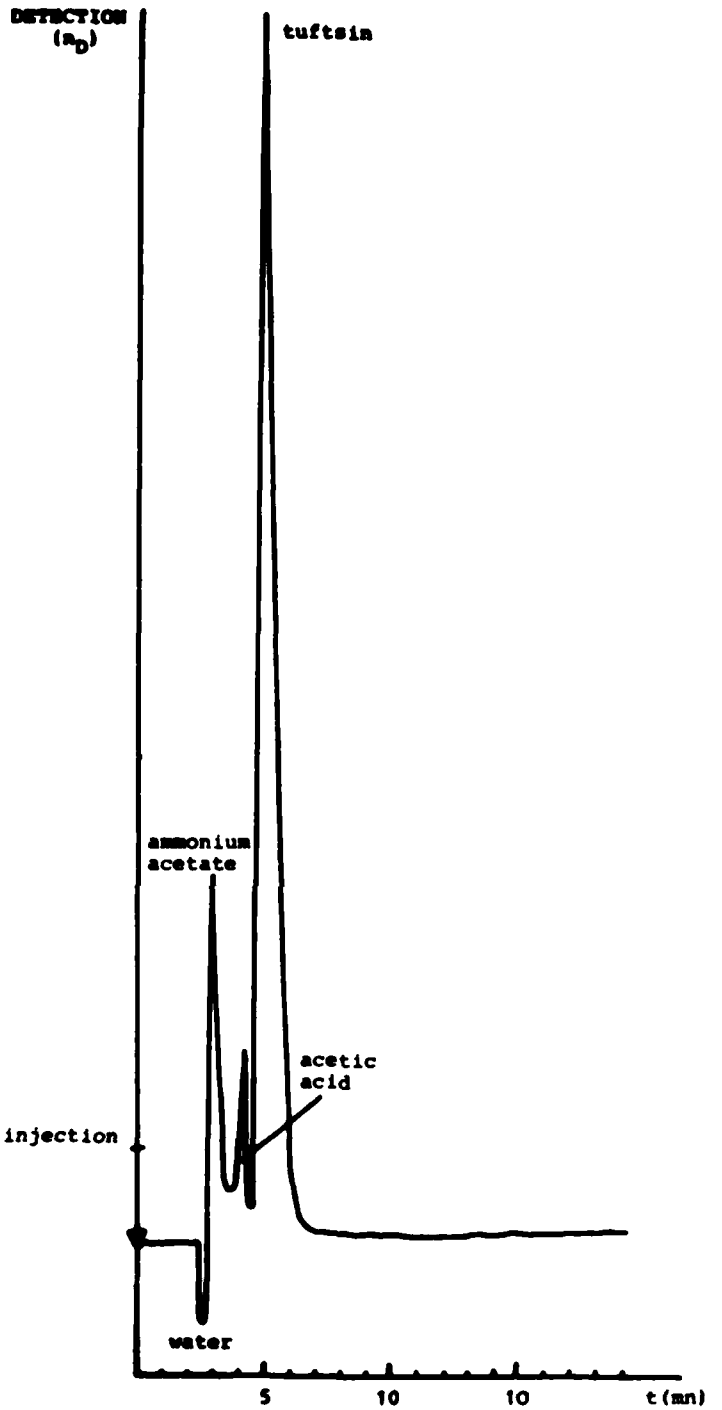


FIGURE 1. Analytic HPLC chromatography of tuftsin (fraction A). Column C 18 (Waters). Solvent $PO_4H_2NH_4$ 0.1 *M* (1.1 ml/min). Pressure 1500 psi.

Synthesis of 50 g of pure tuftsin[23] was carried out according to SCHEME 1. We used groups that are susceptible to hydrogenolysis for α carboxylic and guanidine protection of arginine, for ϵ-amino function of lysine and for the α-amino group of threonine. *Tert*-butyloxycarbonyl (BOC) group was used for α-amino protection during the growth of the peptide chain and was cleaved with TFA and with a mixture of

TFA-acetic acid[24] after introduction of lysine to minimize the loss of the N-ε-amino protecting group. After removal of all protecting groups by hydrogenolysis using Pd/$BaSO_4$ 10% as catalyst, tuftsin was purified by preparative HPLC. Purity of samples was ascertained by elemental analysis, TLC, amino-acid analysis, and analytical HPLC. (FIG. 1).

Reproducibility of the method and purity of samples of tuftsin obtained by this way are important in the preparation of large amounts of tuftsin.

SYNTHESIS OF TUFTSIN-RELATED COMPOUNDS

Previous structure/function studies of tuftsin analogues showed that the character of the amino acid in position 1 seems not to be of great importance in providing specific binding of the tuftsin molecule with cell receptors and antibodies and could be, in some cases, replaced without affecting its biological activities. We undertook the synthesis of various tuftsin-related compounds, modified at position 1. We will describe only the synthesis of compounds exhibiting significant biological activity as compared to tuftsin (TABLE 1).

TABLE 1. Tuftsin Synthetic Analogues Exhibiting Phagocytosis-stimulating Activity

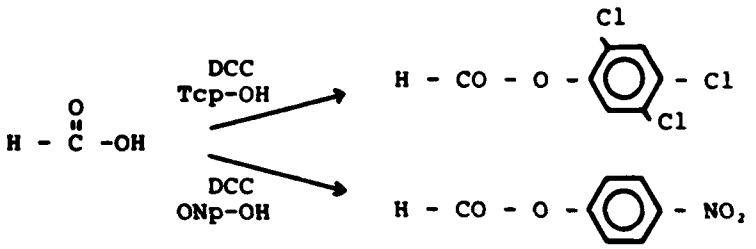
Met-Lys-Pro-Arg	(Met-1)-Tuftsin
For-Met-Lys-Pro-Arg	(For-Met-1)-Tuftsin
For-Thr-Lys-Pro-Arg	For-Tuftsin
Ind[a]-Lys-Pro-Arg	(Ind-1)-Tuftsin
Ind-Thr-Lys-Pro-Arg	Ind-Tuftsin

[a]Ind: indomethacin.

The peptide Leu-Lys-Pro-Arg is highly potent in stimulating phagocytosis of polymorphonuclear leukocytes.[25] It is well known that methionine and leucine can replace each other in natural analogues of several biologically active peptides. For instance, position 5 in gastrin is occupied by methionine in porcine tissues,[26] while leucine occurs in human gastrin.[27] A further illustration is provided by the enkephalins,[28] one having a methionine and another a leucine at their COOH-terminus. Similarities between solubility and physical properties of these two amino-acid residues are also suggestive. For these reasons, we expected that replacement of leucine by methionine in the tuftsin analogue Leu-Lys-Pro-Arg should result in an active compound.

Synthesis of Met-Lys-Pro-Arg was carried out from Z-Met-ONp[29] and the partially deprotected tripeptide Lys (Z)-Pro-Arg (NO_2) OBzl. Hydrogenolysis[30] of the resulting compound in a mixture of dimethylformamide, diisopropylethylamine, and water in the presence of Pd/$BaSO_4$ 10% and purification by HPLC gave Met-Lys-Pro-Arg.

It was recently reported that, in addition to promoting cell locomotion, a further biological function of leukoattractants (formyl-peptides) may be their capacity to render complement receptors more freely available, thereby increasing the magnitude of adhesion of phagocytic cells to opsonized particles.[31] Thus, we prepared the peptide For-Met-Lys-Pro-Arg, as a possible leukoattractant and phagocytosis-stimulating peptide. Its synthesis was performed by condensing Boc-Met-OSu[32] with the tripeptide Lys (Z)–Pro-Arg (NO_2) OBzl to yield the tetrapeptide Boc-Met-Lys (Z)-Pro-Arg



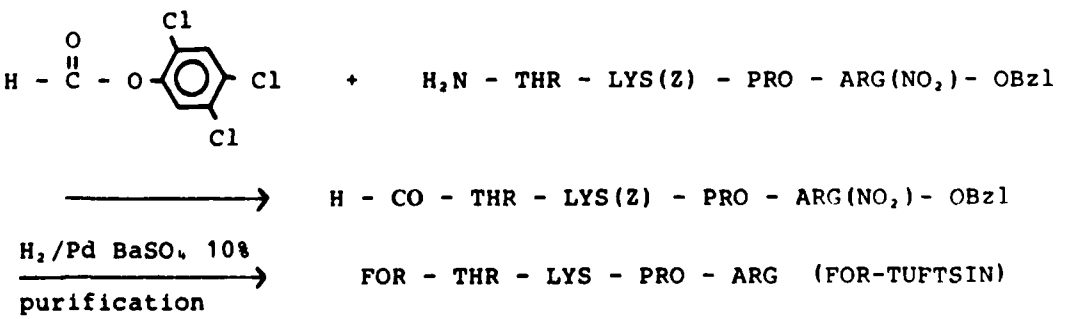
SCHEME 2. Synthesis of formyl active esters.

(NO_2) OBzl. Partial deprotection by a mixture of trifluoroacetic acid, acetic acid 7:3, yielded Met-Lys (Z)-Pro-Arg (NO_2) OBzl. Formylation of this peptide was accomplished using active esters of formic acid (scheme 3), either *p*–nitrophenyl formate, or 2,4,5-trichlorophenyl formate obtained as illustrated in SCHEME 2. This way of formylating peptides at their NH_2-terminus proved to be highly satisfactory[33] and the protected peptide For-Met-Lys (Z)–Pro-Arg-(NO_2) OBzl was secured pure and in very good yield. It was deprotected by hydrogenolysis and purified as previously described. For-Thr-Lys-Pro-Arg was also synthesized, according to SCHEME 3.

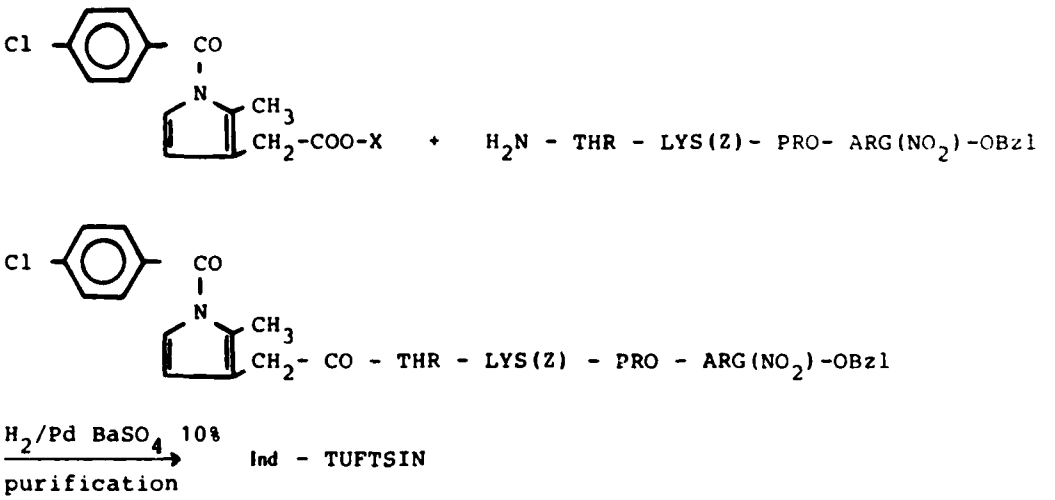
Indomethacin is involved in inhibition of prostaglandin biosynthesis and in a phenomenon related to the secretion of acid hydrolases by mononuclear phagocytes in chronic inflammation. It was also reported that indomethacin enhanced polymorphonuclear-stimulated migration, possibly due to selective inhibition of prostaglandin synthesis.[34] On the other hand, the potential use of indomethacin as an immunoadjuvant has been confirmed.[35] For these reasons, we undertook the synthesis of Indomethacyl-Lysyl-Prolyl-Arginine, (Ind-1)-tuftsin and of Indomethacyl-Threonyl-Lysyl-Prolyl-Arginine, (Ind-tuftsin). This compound was prepared by reaction of the active ester of indomethacin with the partially protected tetrapeptide Thr-Lys (Z)-Pro-Arg (NO_2) OBzl (SCHEME 4). The peptide (Ind-1)-tuftsin was secured by condensing indomethacin-active ester with the partially protected tripeptide Lys (Z)–Pro-Arg (NO_2) OBzl. Both peptides were fully deprotected by hydrogenolysis using Pd/$BaSO_4$ 10% as catalyst and purified by column chromatography on silica gel.

SYNTHESIS OF AN IgG DECAPEPTIDE FRAGMENT

Tuftsin is located in the Fc portion of the heavy chain of leukokinin, at residues 289–292.[36] Tuftsin is liberated from the carrier molecule by two specific enzymes: (1)



SCHEME 3. Synthesis of For-tuftsin.

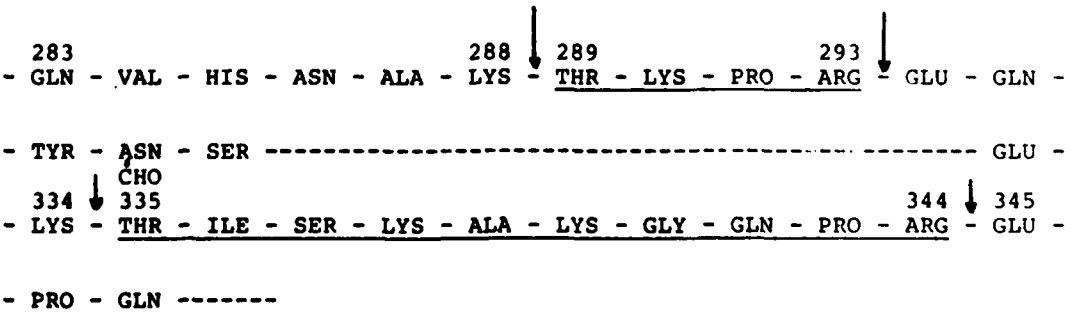


SCHEME 4. Synthesis of Ind-tuftsin. X = 2,4,5-trichlorophenyl, 4-nitrophenyl.

tuftsin endocarboxypeptidase, a splenic enzyme, cleaves at the arginine 292–glutamic acid 293 bond; and (2) leukokininase cleaves at the Lys 288–threonine 289 bond.

Careful examination of the human IgG sequence reveals that an analogous enzymatic cleavage between Lys 334 and threonine 335 and between arginine 344 and glutamic acid 345 would lead to liberation of the fragment decapeptide 335–344 of IgG (SCHEME 5), Thr-Ile-Ser-Lys-Ala-Lys-Gly-Gln-Pro-Arg. It is interesting to note that this decapeptide contains in its sequence the tetrapeptide Gly-Gln-Pro-Arg, named rigin, which has been shown to stimulate phagocytosis of PMN as well as tuftsin.[37,38]

It was tempting to assume this decapeptide could exist in the body and to hypothesize some biological role for it. Considering the above, for the enzymatic liberation of this fragment decapeptide, it must be located on the external surface of immunoglobulin to be accessible to enzymatic actions. Recently, S. Fraga proposed a way of predicting the antigenic determinants of a protein[39] from its amino-acid sequence by a modified Hopp-Woods method,[40] using both hydrophilicity and recognition factors. These factors gave information for the individual amino acids in their



SCHEME 5. Sequence in the C_H regions of human γ chains. CHO represents carbohydrate. Arrows indicate points of specific enzymatic cleavage that lead to liberation of tuftsin and that could lead to liberation of the fragment 335–344 of the carrier molecule.

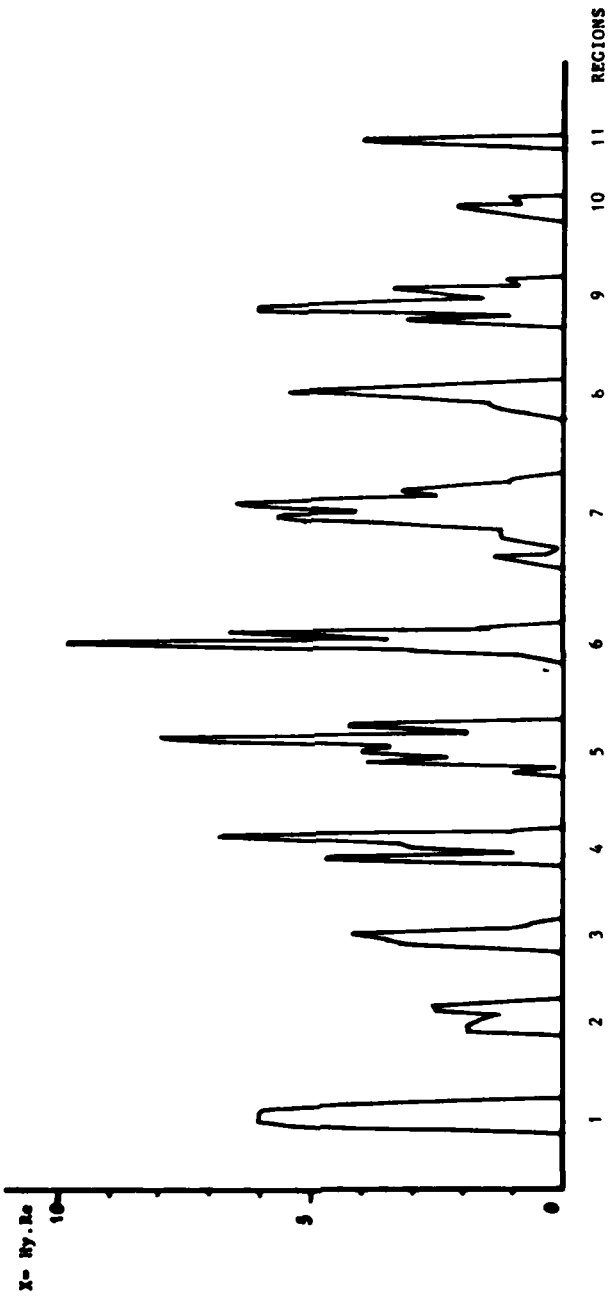


FIGURE 2. Profile of the C_H region of human IgG. (X is the product between hydrophilicity (Hy) and recognition (Re).)

hexapeptide surroundings and were evaluated by a semiempirical method. According to Fraga, this procedure leads to very satisfactory predictions of the antigenic determinants, eliminating most of the uncertainties in the original Hopp-Woods method. We applied Fraga's method of calculation to the CH region of the γ-human-IgG protein (FIGURE 2) by calculating a modified recognition factor as the difference between the mean of Fraga's values and the individual amino acid value. Regions with negative hydrophilicity will never correspond to regions of antigenic activity and regions with negative recognition factor will be assumed to lack antigenic activity. We found 11 substantial determinants[a] corresponding to region 1 (residues 210–215), region 2 (residues 217–223), region 3 (residues 246–250), region 4 (residues

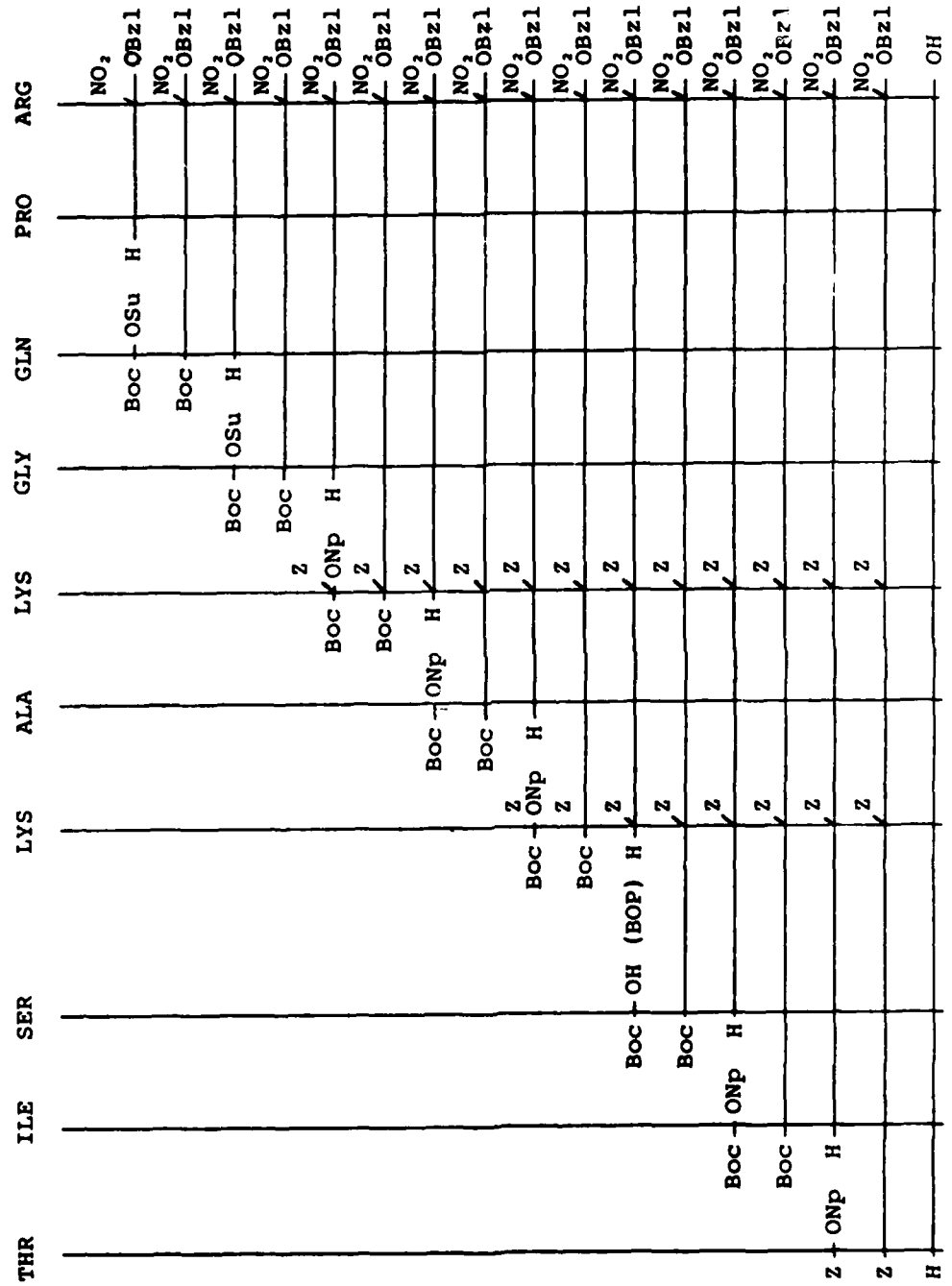
TABLE 2. Amino Acid Sequences of Potential Antigenic Determinants in The C_H Region of Human IgG

	210 215
Region 1	-Lys-Val-Asp-Lys-Arg-Val-
	217 223
Region 2	-Pro-Lys-Ser-Cys-Asp-Lys-Thr-
	246 250
Region 3	-Lys-Pro-Lys-Asp-Thr-
	266 272
Region 4	-Val-Ser-His-Glu-Asp-Pro-Gln-
	285 295
Region 5	-His-Asn-Ala-Lys-Thr-Lys-Pro-Arg-Glu-Gln-Gln-
	314 321
Region 6	-Leu-Asp-Gly-Lys-Gln-Tyr-Lys-Cys-
	331
Region 7	-Pro-Ile-Glu-Lys-Thr-Ile-Ser-Lys-Ala-Lys-Gly-Gln-Pro-Arg-Glu-Pro-
	353 359
Region 8	-Pro-Ser-Arg-Asp-Gln-Leu-Thr-
	384 391
Region 9	-Asn-Asp-Gly-Glu-Pro-Glu-Asn-Tyr-
	398 401
Region 10	-Leu-Asp-Ser-
	410 412
Region 11	-Leu-Thr-Val-

266–272), region 5 (residues 285–295 containing tuftsin at residues 289–292), region 6 (residues 314–321), region 7 (residues 331–346 containing the fragment decapeptide of IgG 335–344), region 8 (residues 353–359), region 9 (residues 384–391), region 10 (residues 398–401), and region 11 (residues 410–412) (TABLE 2). It is interesting to note that both tuftsin and the fragment decapeptide we synthesized are in the regions of potential antigenic determinants. Similarities in sequences of these natural fragments that could have biological properties are also of some interest.

The theorical prediction of protein antigenic determinants should be based on calculations with consideration of the tertiary structure and the aqueous environment. But with this simplified method, and taking account of both hydrophilic and

[a]Calcutations have been made with a computer program written by Professor B. Castro considering Fraga's data.



SCHEME 6. Synthesis of the IgG decapeptide fragment 335–344.

recognition factors, a rather attractive and simple method is available and appears to be promising.

Synthesis of the decapeptide fragment 335–344 was performed according to SCHEME 6. The peptide was built stepwise with active esters as reactive derivatives of the constituent amino-acid residues. For the coupling of serine, benzotriazolyloxytris (dimethylamino) phosphonium hexafluorophosphate (BOP)[41] was used. Final and total deprotection of the peptide was performed by hydrogenolysis in the presence of $Pd/BaSO_4$ 10% as catalyst. The resulting peptide was purified by chromatography on silica gel and by preparative HPLC.

These synthesized peptides were first evaluated by their capacity to stimulate *in vitro* phagocytosis of human PMN cells against *Staphylococcus aureus* (TABLE 3). Phagocytosis assays were carried out by the method of Najjar and Costantopoulos. It can be seen from TABLE 3 that the peptides synthesized presented some phagocytosis-

TABLE 3. Phagocytosis-stimulating Activity of Human PMN by Tuftsin, Analogues, and by an IgG Decapeptide Fragment

	c μg/ml	I[a]
Plasma		45
Tuftsin	0.05	65
	0.1	70
(Met-1)-Tuftsin	0.05	60
	0.1	65
(For-Met-1)-Tuftsin	0.05	71
	0.1	71
	0.2	70
For-Tuftsin	0.05	65
	0.1	68
(Ind-1)-Tuftsin	0.05	60
	0.1	70
Ind-Tuftsin	0.05	65
	0.1	70
	0.2	70
IgG decapeptide fragment	0.05	55
	0.1	60
	0.2	62

[a]I: number of cells containing more than 20 particles/100 cells. At least 800 cells had been scored for each experiment

stimulating activity. (For-Met-1)-tuftsin and Ind-tuftsin seem to be the two more potent analogues we have. All these compounds have been tested in various immunological experiments, by I. Florentin. The results of her findings will be presented in another paper.

An inherent difficulty in our planned experiments concerning the IgG decapeptide fragment was caused by our ignorance of the possible physiological activities of this peptide. But availability of this compound increases the hope that more light will be shed on its possible biological role.

SUMMARY

The preparation of large amounts of tuftsin (50 g) as well as purification by preparative HPLC are presented. Synthesis of several analogues exhibiting significant

phagocytosis-stimulating activity of human PMN are described and discussed. On the other hand, the synthesis of a natural human IgG decapeptide fragment 335–344 that could be liberated from the carrier molecule by the two specific enzymes responsible for release of tuftsin from the same carrier molecule is presented. This decapeptide fragment, Thr-Ile-Ser-Lys-Ala-Lys-Gly-Gln-Pro-Arg, showed interesting activity in stimulating phagocytosis of human polymorphonuclear leukocytes.

ACKNOWLEDGMENTS

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Elongated Tuftsin Analogues—Synthesis and Biological Investigation

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Recently, we found[1-5] that a tuftsin[d] analogue with a double linear peptide sequence (tuftsinyltuftsin-octapeptide: L-threonyl-L-lysyl-L-prolyl-L-arginyl-L-threonyl-L-lysyl-L-prolyl-L-arginine) showed anticancer activity against murine leukemia cells L 1210 in DBA/2 mice. This effect was much stronger than that found with tuftsin, because of the low possibility of formation of the inhibitory tripeptide (L-lysyl-L-prolyl-L-arginine) tuftsin inhibitor.[1,2]

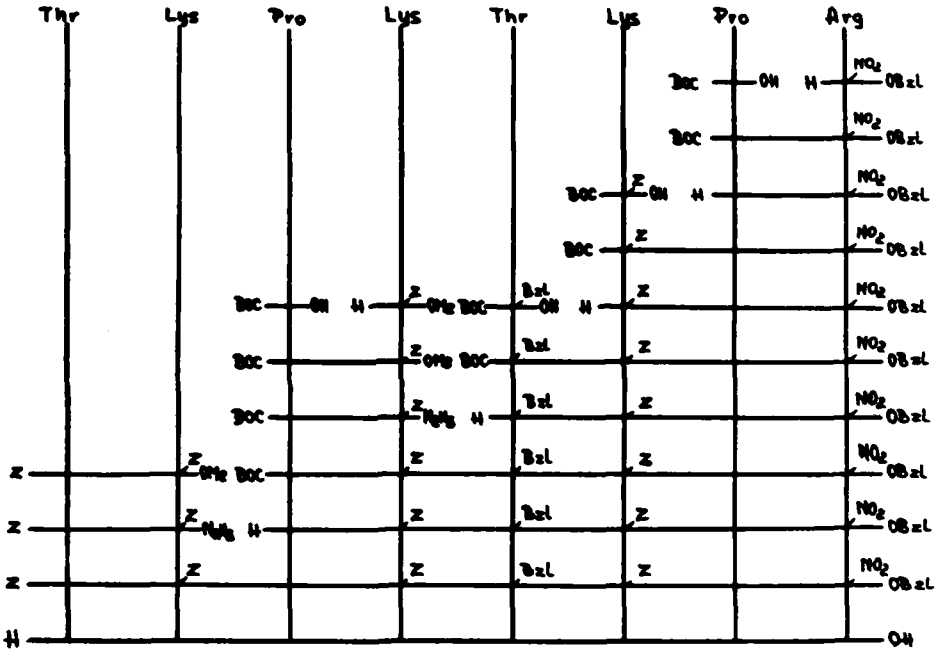
On this basis, we performed the synthesis of similar tuftsin analogues with an elongated peptide chain: L-threonyl-L-lysyl-L-prolyl-L-lysyl-L-threonyl-L-lysyl-L-prolyl-L-arginine (I), ([Lys[4]]-tuftsinyltuftsin), L-threonyl-L-lysyl-L-prolyl-L-lysyl-L-threonyl-L-lysyl-L-prolyl-L-lysine (II), L-analyl-L-lysyl-L-threonyl-L-lysly-L-prolyl-L-arginyl-L-glutamyl-L-glutamine (III—fragment 287–294 of the γ-globulin heavy chain of human blood serum EU), L-arginyl-L-threonyl-L-lysyl-L-prolyl-L-arginine (IV—Arg-tuftsin), L-prolyl-L-arginyl-L-threonyl-L-lysyl-L-prolyl-L-arginine (V—Pro-Arg-tuftsin), and L-lysyl-L-prolyl-L-arginyl-L-threonyl-L-lysyl-L-prolyl-L-arginine (VI—Lys-Pro-Arg-tuftsin).

It is clear that compounds I and II are tuftsin analogues with double peptide sequence and compound III represents residues (287–294) of the natural fragment of the γ-globulin heavy chain that includes the tuftsin fragment. However, the structure of compounds IV-VI were formed by consecutive elongation of the tuftsin chain, from the NH_2-terminal with selected fragments of the tetrapeptide tuftsin. The synthesis of the above-mentioned peptides (I–VI) was aimed at testing the effect of such modification on (1) phagocytosis stimulation and (2) anticancer properties. Moreover, it was especially interesting to examine the effect on phagocytosis of peptides IV–VI, that is, the compounds that were elongated with intermediate sequences of tuftsin itself. The

[d]Tuftsin is the tetrapeptide L-threonyl-L-lysyl-L-prolyl-L-arginine.

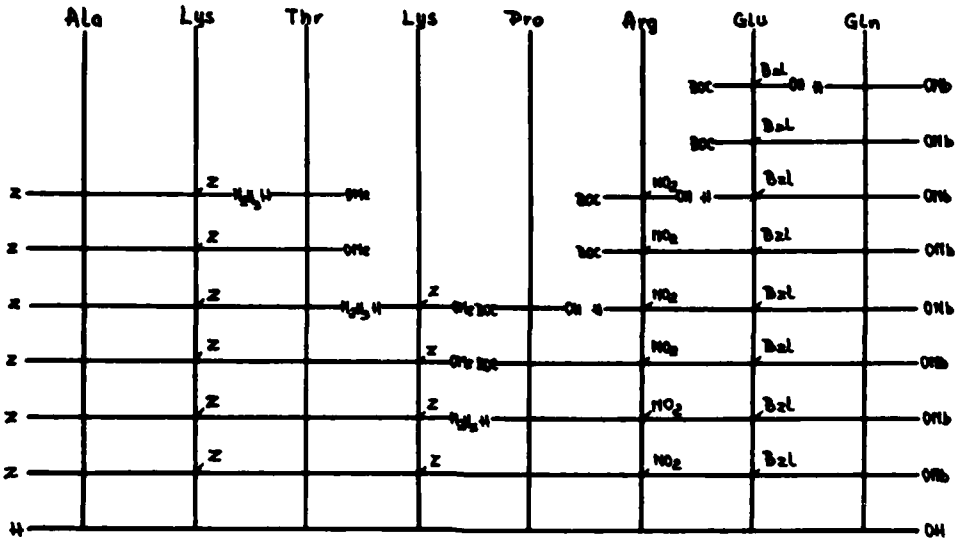
purpose of these investigations stems from earlier results with the octapeptide tuftsinyltuftsin[1,2,4] which failed to stimulate phagocytosis as contrasted with the stimulatory effect of the monomer. Therefore, we examined the phagocytosis-stimulating properties of these elongated tuftsin analogues: Arg-tuftsin (IV), Pro-Arg-tuftsin (V), and Lys-Pro-Arg-tuftsin (VI). This enabled us to study the effect on phagocytosis of the successive elongation of the peptide chain in the series from tuftsin to tuftsinyltuftsin, upon the influence of this modification on the phagocytosis process.

The peptides I–IV were synthesized by the classical method. Compounds I–III were obtained by condensation of the peptide fragments using the azide method (according to SCHEMES 1 and 2). In the case of peptides I and II, the (2 + 2 + 4) condensation was carried out, and for peptide III, the (4 + 4) condensation was carried out. Peptide IV was obtained by dicyclohexylcarbodiimide (DCC) method according to the "step-by-step" system (SCHEME 3). During the synthesis of peptides



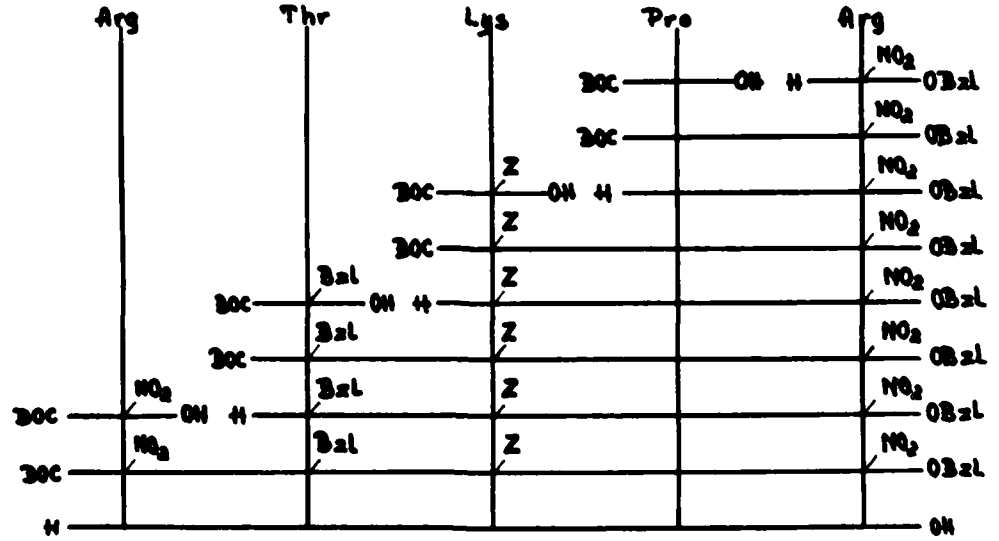
SCHEME 1. Synthesis of [Lys[4]] tuftsinyltuftsin (I).

I–III, the NH_2-terminal amino groups were blocked by benzyloxycarbonyl compound (Z), whereas in the case of peptide IV, by *tert*-butyloxycarbonyl compound (BOC). The remaining α-amino groups of the corresponding amino acids were protected by BOC, which in each case was removed by trifluoroacetic acid (TFA) in dichloromethane (1:1) or 4 *N* hydrogen chloride in dioxane. Bifunctional amino acids were used as N^{ϵ}-Z-L-lysine (H-Lys(Z)-OH, O^{δ}-benzyl-L-glutamic acid (H-Glu(OBzl)-OH), O^{β}-benzyl-L-threonine (H-Thr(OBzl)-OH) and N^{G}-nitro-L-arginine (H-Arg(NO_2)-OH). The COOH-terminal carboxyl groups were protected by benzyl (Bzl) (in peptides I, II, and IV) and 4 nitrobenzyl (Nb) (in peptide III) esters. All the blocked di-, tri-, tetra-, penta-, hexa- and octapeptides were obtained by the DCC mixed anhydride or azide methods. The blocking groups were removed by catalytic hydrogenation in the presence of palladium on barium sulfate and PD($BaSO_4$), and the free peptides obtained (I–IV) were purified by column chromatography on Sephadex G-25.

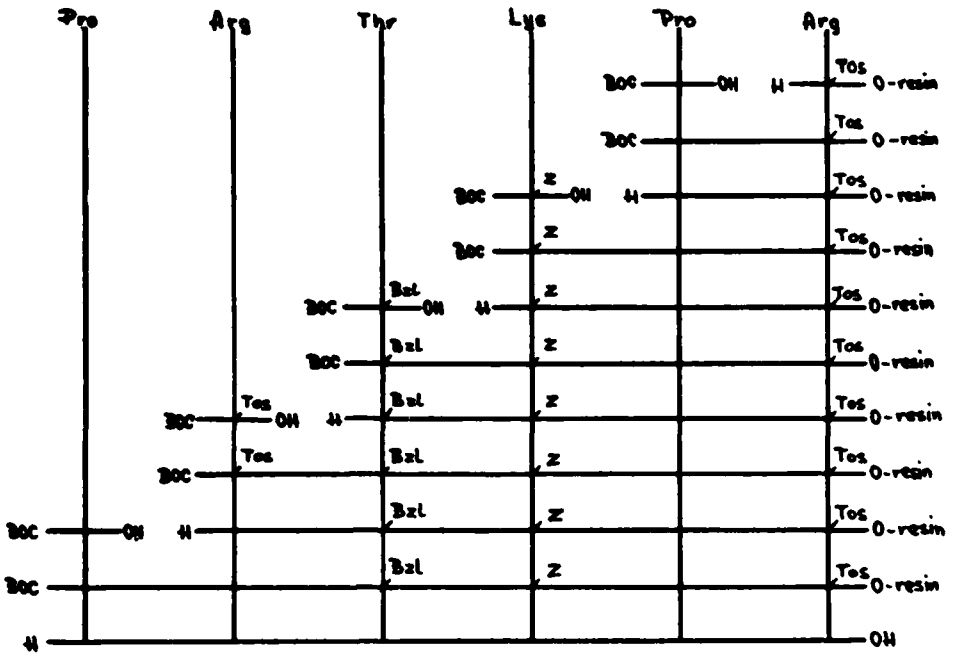


SCHEME 2. Synthesis of fragment 287–294 of γ-globulin heavy chain from human blood serum (III).

Peptide V and VI were synthesized by the Merrifield method (SCHEME 4) using a polystyrene divinylbenzene copolymer and DCC as the condensing agent. All α-amino groups of the corresponding amino acids were protected by BOC, which was removed by TFA in dichloromethane. Bifunctional amino acids H-Lys-(Z)-OH, Thr-(OBzl)-OH, and N^G-tosyl-L-arginine (H-Arg(Tos)-OH were purchased from Bachem Inc., U.S.A. The final peptides were cleaved and simultaneously deprotected using trifluoromethanosulfonic acid in anisole. The free peptides resulting were purified on Dowex IRC-50 and thereafter on a Sephadex G-25 column. Their homogeneity was checked by thin-layer chromatography (TLC) on silica gel plates and paper electrophoresis 700 V. The purity of the products was further checked by amino-acid analysis and nitrogen determination.



SCHEME 3. Synthesis of Arg-tuftsin (IV).



SCHEME 4. Synthesis of Pro-Arg-tuftsin (V) by the solid-phase method.

BIOLOGICAL OBSERVATIONS *IN VITRO*

Peptides I–VI were subjected to the Constantopoulos and Najjar[6] test for estimation of their effect on the phagocytosis process (TABLE 1). From TABLE 1, it appeared that only Arg-tuftsin (IV) possessed approximately 30% of the tuftsin activity, whereas the remaining elongated tuftsin analogues did not stimulate phagocytosis. The biological activities of the oligopeptides I-III, V, and VI are consonant with our earlier results obtained for tuftsinyltuftsin[1,2] and for other elongated tuftsin analogues, which constituted the fragments of the γ-globulin heavy chain of human blood serum.[2,7] Thus, the data presented in the table indicate clearly that the tuftsin peptides elongated from the NH_2-terminal are inactive. It should be noted that the shortened tuftsin analogue

TABLE 1. Elongated Tuftsin Analogues in the Phagocytosis Test[a]

Peptides	Biological Activity Relative to Tuftsin
Tuftsin	1.0
[Lys[4]]-tuftsinyltuftsin (I)	0.0
Canine tuftsinyltuftsin (II)	0.0
Fragment 287–294 of γ-globulin heavy chain from human blood serum (III)	0.0
Arg-tuftsin (IV)	0.33
Pro-Arg-tuftsin (V)	0.0
Lys-Pro-Arg-tuftsin (VI)	0.0
Tuftsinyltuftsin	0.0[b]
L-lysyl-L-prolyl-L-arginine	inhibitor[b]

[a]The test was carried out according to Constantopoulos & Najjar.[6]
[b]According to Najjar *et al.*[1,2]

L-lysyl-L-prolyl-L-arginine acts as an inhibitor of phagocytosis.[1,2] These data support the thesis that only the tetrapeptide (i.e. tuftsin) possessed optimal phagocytosis-stimulating properties.

BIOLOGICAL OBSERVATIONS *IN VIVO*

The effect of [Lys^4]-tuftsinyltuftsin on the development of a tumor induced by intramuscular inoculation with murine sarcoma virus (MSV) was investigated and

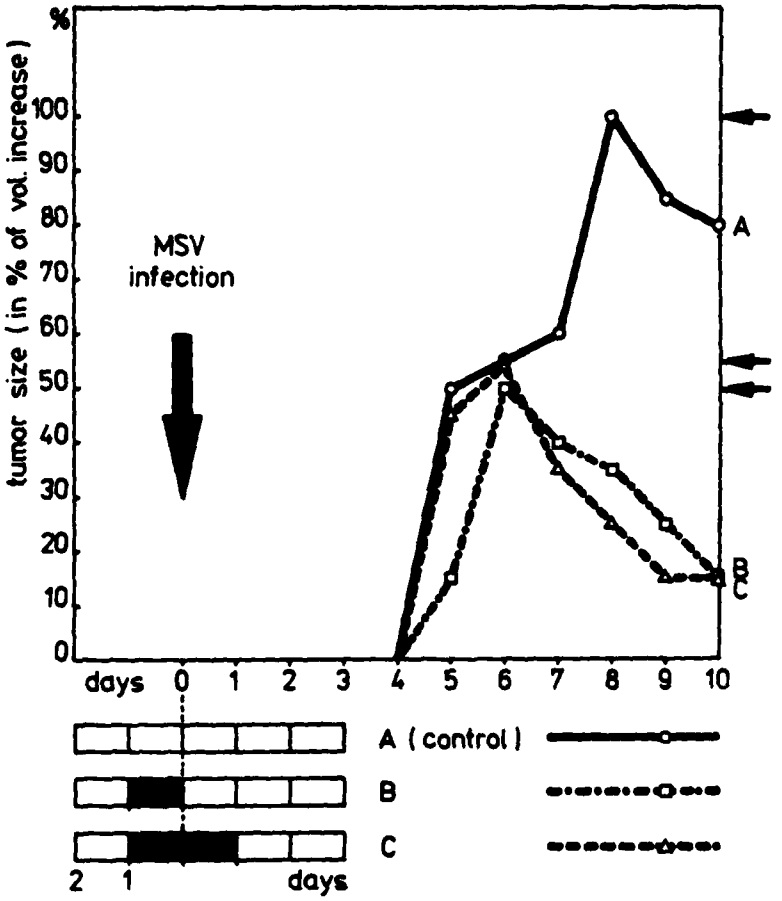


FIGURE 1. Development of MSV tumor after treatment with [Lys^4]-tuftsinyltuftsin, administered i.p. at 10/μg per dose: 24 hr before (B) or 24 hr before and 24 hr after (C) inoculation in comparison with controls (A).

compared with those of tuftsin. Both substances were dissolved in phosphate-buffered saline (PBS) and stored in −20°C until use.

Standard virus inoculum was prepared by intramuscular injection in the left hind leg of 1–2-day-old NMRI mice using 0.05 ml of virus solution (diluted with saline 1:10). On the ninth day, animals were killed by cervical dislocation, and the tumor dissected. The virus extract was prepared from these tumors according to Chanaille *et al.*[8] Other procedures were essentially the same as reported previously.[9] Tumors were produced by inoculating 10-week-old NMRI male mice (20-g body weight) with 0.2

ml of MSV.[10] For inoculation, virus particles were diluted 1:5 with PBS and injected intramuscularly in a 0.2-ml volume into the right thigh.[11]

Tumor development was recorded during 10 days, and its size measured. The size of the tumor was expressed in percent of increase in the volume of the infected thigh, as compared with the volume of the intact thigh.

The peptides were injected intraperitoneally (10 μg per injection) according to the following schedule:

Group B—single injection 24 hr before MSV inoculation;
Group C—two injections, 24 hr before and 24 hr after MSV;
Group D—four injections, 24 hr and 12 hr before and 24 hr and 35 hr after MSV; and
Group E—six injections, 48, 36, 24, and 12 hr before and 12 and 24 hr after MSV.

Control animals (group A) received only PBS injections. Each group consisted of at least 10 animals.

As shown in FIGURE 1 and FIGURE 2 [Lys^4]-tuftsinyltuftsin inhibited tumor growth independently of the schedule of administration. The best results were observed when the peptide was administered twice, 24 hr before and 24 hr after inoculation.

Unexpectedly, the effect of the tuftsin on tumor development was closely related to the schedule of administration. Tuftsin administered once 24 hr before viral inocula-

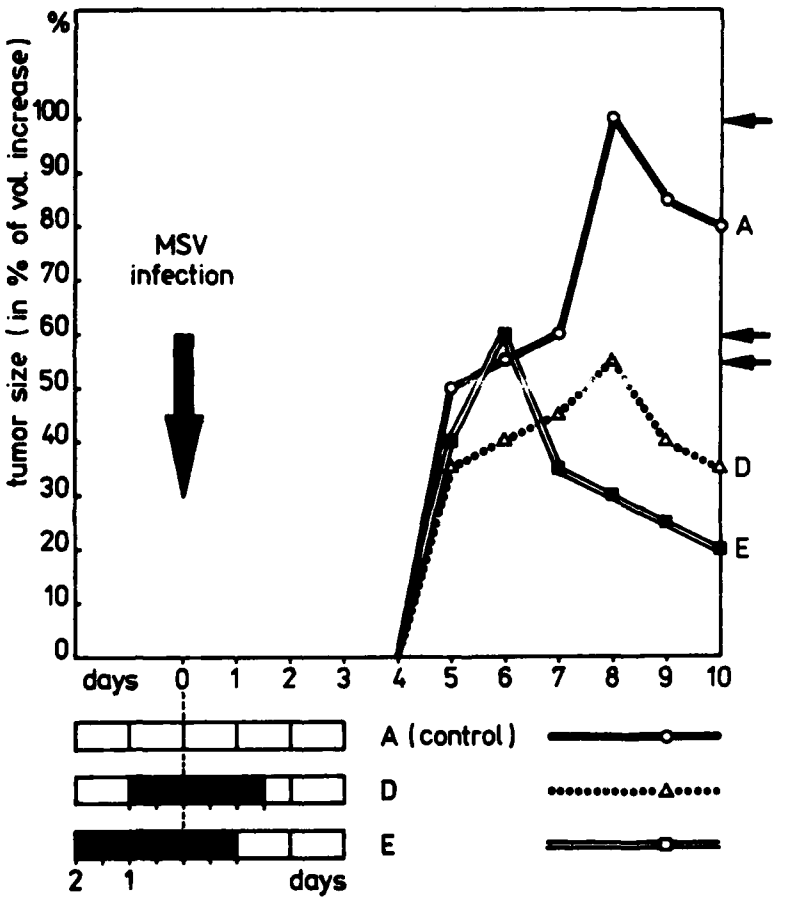


FIGURE 2. Effect of [Lys^4]-tuftsinyltuftsin administered 24 hr and 12 hr before and 24 hr and 36 hr after (D) or 48 hr, 36 hr, 24 hr and 12 hr before and 12 hr and 24 hr after (E) MSV inoculation on tumor growth.

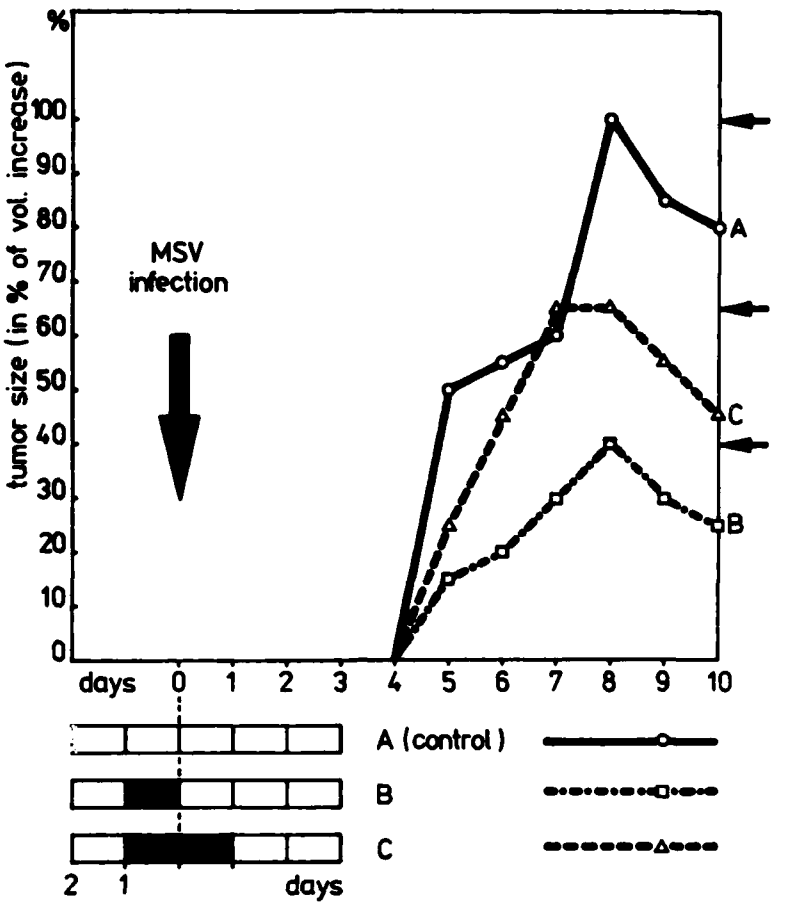


FIGURE 3. Development of MSV tumor after treatment with tuftsin administered i.p. at 10 μg per dose: 24 hr before (B) or 24 hr before and 24 hr after (C) inoculation in comparison with controls (A).

tion delayed the tumor development and markedly reduced its size (FIG. 3). However, when the peptide was administered four times, 24 hr and 12 hr before and 24 hr and 36 hr after inoculation (Schedule D, FIG. 4), tumor development was accelerated and its size was even somewhat greater than in the control group (FIG. 4).

The results reported here indicate that both peptides are able to decrease the size of MSV-induced tumors. The effect of tuftsin depends on the schedule of administration. The best results with tuftsin were obtained when the peptide was administered in single injection 24 hr before virus inoculation. It is of interest to note that on a molar basis, the dose of tuftsin was about 2 times greater than that of [Lys^4]-tuftsinyltuftsin. In future studies, we intend to investigate the effects of both peptides in equal molar doses as well as to extend the level of dosage. It is at present difficult to explain the mechanism of the observed antitumor action of tuftsin and its analogue [Lys^4]-tuftsinyltuftsin. It is possible that this effect of tuftsin or its analogues depends either on a direct or indirect cytotoxic activity of these compounds.[12]

The antiviral activity of the above-mentioned peptides must be also taken into consideration. Our preliminary experiments, not reported here[13] indicated that tuftsin probably possesses antiviral activity. In mice that are infected intranasally with virus herpes hominis type 1 (HSV_1), tuftsin markedly reduced the mortality (up to 80%, depending on the schedule of administration). Similar results were obtained in mice

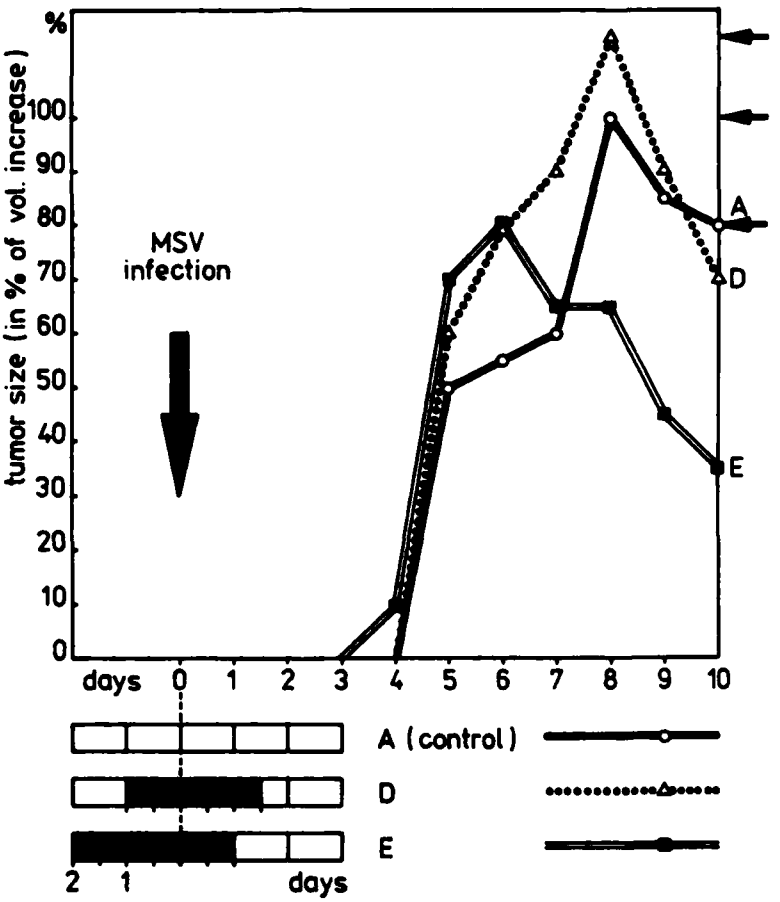


FIGURE 4. Effect of tuftsin administered 24 hr and 12 hr before and 24 hr and 36 hr after (D) or 48 hr, 36 hr, 24 hr, 12 hr before and 12 hr and 24 hr after (E) MSV inoculation on tumor growth.

infected with EMC-virus. The details follow the method described by De Clerq and Luczak 1976.[14] It is of interest to note that [Lys^4]-tuftsinyltuftsin was effective in both of the above-mentioned tests.

These results, however, are of preliminary character and further experiments are necessary to clarify the antineoplastic properties of tuftsin and its analogues.

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NMR Studies of the Conformation of Tuftsin and a Pentapeptide Tuftsin Inhibitor in Solution[a]

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INTRODUCTION

A complete understanding of the mode of action of tuftsin requires a knowledge of its biologically active conformation. As with most peptides, a direct measurement of the active conformation of tuftsin has not been possible, so indirect methods have been employed. Numerous tuftsin analogues have been synthesized, and measurements of activity and of inhibitory properties have led to some conclusions concerning obligatory structural features (reviewed in Siemion & Konopinska[1]). A variety of physical techniques have been used to study the structure of tuftsin in solution. Investigations have been performed on tuftsin as well as tuftsin analogues, in aqueous and nonaqueous solutions. Among the spectroscopic techniques employed have been nuclear magnetic resonance (NMR),[2-4] circular dichroism (cd)[4,5] and infrared (IR).[6] Theoretical calculations have also been carried out.[7,8] In light of the rather short length of tuftsin, it is somewhat surprising that there has been no general agreement on its structure in solution. Among the proposed structures are those involving a β-turn,[4,6] a quasi-cyclic molecule,[7] and a hairpin with split ends.[8] We have employed ^1H and ^{13}C NMR[2] to study the structure of tuftsin and pentapeptide inhibitor, Thr-Lys-Pro-Pro-Arg. Our results indicate that in aqueous solution tuftsin exists in a random conformation, while in dimethyl sulfoxide (Me_2SO), a somewhat more ordered, but as yet undetermined, structure exists. In this paper, I will review our results, as well as the results from other laboratories. In particular, I will show that a wealth of available evidence precludes the possibility that tuftsin exists in a β-turn structure in aqueous solution.

MATERIALS AND METHODS

Protected L-amino acids were purchased from Bachem. Solvents and other chemicals were reagent grade. Deuterium oxide (99.8 and 100%) and Me_2SO-d_6 (99.5 and 99.96%) were from Aldrich.

[a]This work was supported by the National Institutes of Health, the National Science Foundation, and the March of Dimes. The NMR facility of the Francis Bitter Magnet Laboratory at the Massachusetts Institute of Technology, which includes the Bruker HX-270 spectrometer, is supported by the National Institute of Health (RR 00995) and the National Science Foundation (Contract C670).

Synthesis of Peptides

Tuftsin and its inhibitory analogue were synthesized by previously described solid-phase techniques.[9]

Nuclear Magnetic Resonance Spectroscopy

^{13}C spectra at 67.9 MHz and ^{1}H spectra at 270 MHz were acquired on a Bruker HX-270 spectrometer. ^{13}C spectra at 15.1 MHz were from a Bruker WP-60. Sample concentrations were about 0.04 *M* for ^{13}C and 0.01 *M* for ^{1}H. Sample temperature was 37° for samples in Me_2SO-d_6, and for T_1 runs, while other spectra, unless otherwise noted, were acquired at ambient temperatures, (27° to 28°C). T_1 values were acquired by use of the inversion recovery technique.[10] ^{1}H spectra in H_2O were obtained by presaturation of the water resonance.[11] Presaturation, using a variable-length presaturation pulse, was employed to measure saturation transfer in Me_2SO-d_6-5% H_2O.

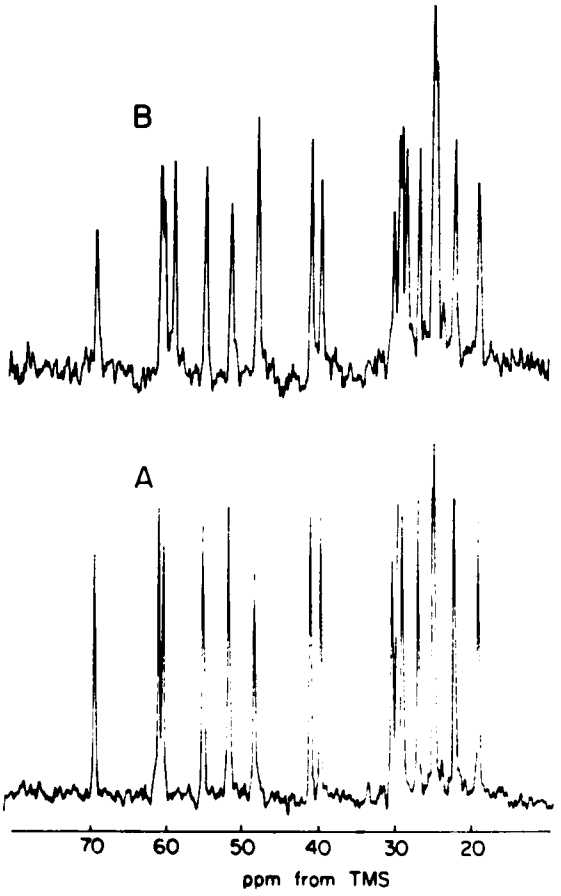

FIGURE 1. ^{13}C spectra of the aliphatic carbons of tuftsin (A) and tuftsin inhibitor (B) at 0.035 *M* in H_2O-10% D_2O, pH 8.7, 25°C. Spectra were acquired at 15.1 MHz (WP-60 spectrometer) by using 90° pulses (21 μsec) and a 1-sec repetition rate. For each spectrum, 70,000 pulses were acquired. The small peak observed at ~23 ppm in each spectrum is due to the methyl carbon of acetate. Its intensity is low due to its very long relaxation time and low nuclear Overhauser enhancement. Reprinted from Blumenstein *et al.*[2] with permission. Copyright 1979, American Chemical Society.

RESULTS

In FIGURE 1 are ^{13}C spectra at 15.1 MHz of tuftsin and tuftsin inhibitor in H_2O. Resonances could be assigned by comparison of observed chemical shifts with chemical

shifts of amino acids in model peptides, and from pH titrations. The titration curves are shown in FIGURES 2 and 3. Several observations can be made from these data: (1) Corresponding carbons in the two peptides have chemical shifts that are almost identical; (2) The measured pKa values of 7.1 for the α-amino group, 3.0 for the α-carboxyl group, and 10.0 for the ϵ-amino group are very close to values observed with random coil peptides;[12] (3) There are no through-space titration effects, only resonances due to carbons within a few bonds of the titrating group undergo shifts; (4) The chemical shifts of the Pro β and γ resonances, and the lack of resonance doubling, indicate that the X-Pro bonds exist totally in the *trans* configuration.[13]

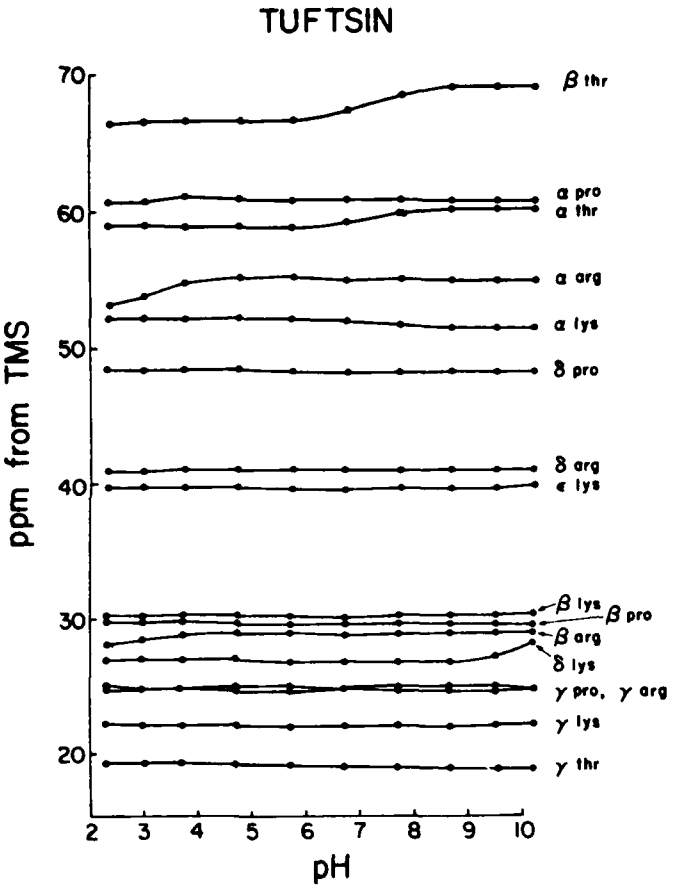

FIGURE 2. ^{13}C chemical shifts of aliphatic carbons of tuftsin as a function of pH. Solution conditions and spectral parameters are as described in FIGURE 1. 10,000–30,000 pulses were acquired for each spectrum. Reprinted from Blumenstein *et al.*[2] with permission. Copyright 1979, American Chemical Society.

Spectra of tuftsin and tuftsin inhibitor in Me_2SO contained resonances that were considerably broader than those observed in H_2O. The quality of the ^{13}C spectra at 15.1 MHz was fairly poor, but at 67.9 MHz good spectra were obtained (FIG. 4). The ^{13}C chemical shifts of tuftsin and tuftsin inhibitor in water and in Me_2SO-d_6 are listed in TABLE 1.

In water, there were no significant shift differences between corresponding carbons

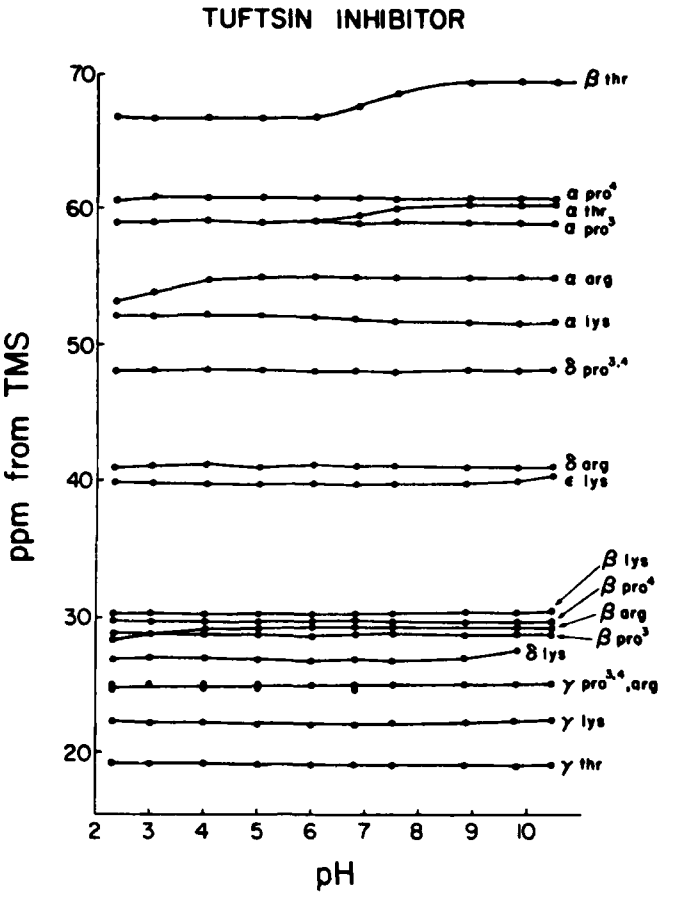

FIGURE 3. ^{13}C chemical shifts of tuftsin inhibitor as a function of pH. Reprinted from Blumenstein *et al.*[2] with permission. Copyright 1979, American Chemical Society.

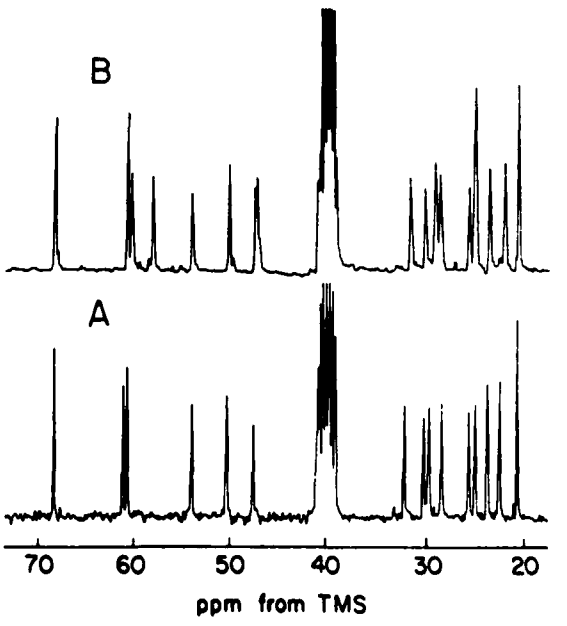

FIGURE 4. ^{13}C spectra of tuftsin (A) and tuftsin inhibitor (B) at 0.03–0.04 *M* in Me_2SO-d_6, 37°C. Spectra were acquired at 67.9 MHz (HX-270 spectrometer) by using 90° pulses (21.5 μsec) and a 1-sec repetition rate. For (A) 7,000 pulses were acquiring and for (B) 40,000 pulses. The acetate peak appearing at 23 ppm is much larger than the peak observed in water since the relaxation time of the acetate is greatly reduced in Me_2SO. On an expanded scale, the peaks due to the carbon of lysine and the carbon of arginine can be observed in this region. Reprinted from Blumenstein *et al.*[2] with permission. Copyright 1979, American Chemical Society.

TABLE 1. ^{13}C Chemical Shifts of Tuftsin and Tuftsin Inhibitor (ppm) from Me_4Si[a]

	H_2O (pH 8.6)		Me_2SO-d_6	
	Tuftsin	Inhibitor	Tuftsin	Inhibitor
Thr	69.4	69.5	68.4	68.3
Pro[b]	61.1	61.0	61.4	60.5
Thr	60.5	60.6	61.1	61.0
Pro[c]		59.2		58.4
Arg	55.2	55.1	54.3	54.3
Lys	51.8	51.7	50.7	50.5
Pro[b]	48.4	48.2	47.8	47.7
Pro[c]		48.2		47.5
Arg	41.1	41.2	41.2	41.2
Lys	39.8	39.9	39.5	39.5
Lys	30.4	30.3	{ 32.4	{ 31.9
Pro[b]	29.7	29.6	{ 30.4	{ 30.4
Arg	29.1	29.3	{ 29.9	{ 29.3
Pro[c]		28.7		29.3
Lys	27.0	27.0	28.6	28.9
Pro[b] }	25.0	25.1	25.8	25.9
Arg }	24.9	24.9	25.2	25.4
Pro[c]		25.1		25.4
Lys	22.2	22.2	22.7	22.3
Thr	19.1	19.1	20.9	20.9
(0=)C Arg	178.7	178.6	{ 175.0	{ 175.4
(0=)C Thr	175.1	175.3	{ 174.6	{ 174.4
(0=)C Lys	173.6	173.7	{ 171.6	{ 171.3
(0=)C Prob	172.3	172.5	{ 170.7	{ 170.7
(0=)C Pro[c]		171.8		{ 170.5
guanido Arg	157.2	157.2	158.4	158.4

[a] Braces indicate values not assignable to specific residues.

[b] Designates Pro^3 of tuftsin and the corresponding Pro^4 of the inhibitor.

[c] Designates Pro^3 of inhibitor. (Reprinted from Blumenstein *et al.*[2] with permission. Copyright 1979, American Chemical Society.)

in the two peptides. In Me_2SO, most carbons in the peptides had very similar shifts, but differences of 0.4 to 1.0 ppm were observed for several carbons. In particular, resonances due to the α-carbon of the proline adjacent to the COOH-terminal residue, β-carbons, the γ-carbon of lysine, and carbonyl carbons of arginine and threonine, all had different chemical shifts in the inhibitor than in tuftsin, when spectra were recorded in Me_2SO. These shift changes are indicative of conformational differences between tuftsin and the inhibitor in Me_2SO. The shift changes are not likely due to primary structure effects, since in water identical shifts are seen for the two peptides.

Relaxation time (T_1) measurements for tuftsin and tuftsin inhibitor in water and in Me_2SO were undertaken at both 15.1 and 67.9 MHz. The measured values, expressed as NT_1, where N is the number of protons bonded to the observed carbon, are listed in TABLE 2. There are no significant differences between tuftsin and tuftsin inhibitor. Both peptides show reduced T_1 values in Me_2SO as compared to water, an observation consistent with the greater line widths seen in Me_2SO. The overall correlation time for each peptide is about 10^{-10}sec in water and 10^{-9}sec in Me_2SO. In water, relaxation times increase significantly for carbons down the side chain of Lys and Arg, while this effect is smaller in Me_2SO. The γ carbons of the prolines have increased T_1 values relative to those of the β and δ carbons because of interconversion between various

puckered forms of the proline ring.[14] The increased T_1 values at 67.9 MHz follow the expected frequency dependence of T_1,[15] with the greater differences in Me_2SO arising from the increased correlation time.

The 270 MHz ^1H spectra of tuftsin and tuftsin inhibitor were very similar to each other. The aliphatic region of the spectra in D_2O are shown in FIGURE 5. Assignments were based on predicted positions of amino-acid resonances, single frequency decoupling, and pH titration. The spectra in Me_2SO (not shown) were similar to those in D_2O, but as with the ^{13}C spectra, the spectral resolution was decreased due to broader lines.

Much information on peptide conformation can often be obtained from studies of peptide NH resonances. In Me_2SO, two such resonances were observed with each peptide. The further downfield resonance, which broadened as the temperature was raised, could be assigned to the Lys residue, since this spectral behavior is characteristic of an amide proton on a residue adjacent to an amino-terminal residue. The other NH was of course due to Arg. In water, both NH protons were observable at low temperature, but at room temperature the Lys NH was broadened beyond detection. Protons from the guanido group of Arg were visible only at low temperatures, and data on these protons were not used in considering possible conformations.

The chemical shifts, temperature dependence of the chemical shifts, and coupling

TABLE 2. Relaxation Time (NT_1) Values in Seconds of Aliphatic Carbon Atoms in Tuftsin and Tuftsin Inhibitor[a]

	H_2O (pH 5)				Me_2SO-d			
	Tuftsin		Inhibitor		Tuftsin		Inhibitor	
	15 MHz	68 MHz	15 MHz	68 MHz	15 MHz	68 MHz	15 MHz	68 MHz
βThr	0.51	0.73	~0.50	0.71	0.15	0.28	0.14	0.30
αPro[b]	0.32	0.53	0.26	0.46	0.08	0.19	0.08	0.17
αThr	0.49	0.64	0.39	0.44		0.27		0.27
αPro[c]							0.05	0.16
αArg	0.41	0.70	0.42	0.58	0.06	0.16	0.05	0.18
αLys	0.32	0.47	0.29	0.45	0.05	0.19	0.05	0.18
δPro[b]	0.38	0.50	~0.25	0.61	0.06	0.26	0.04	0.24
δPro[c]				0.61				0.28
δArg	0.88	1.10	0.66	1.10	No[d]	0.24	NO	~0.20
εLys	1.30	2.10	1.40	2.00	NO	0.44	NO	~0.30
βLys	0.60	0.72	0.32	0.58	0.06	0.28	0.06	0.26
βPro[b]	0.44	0.62	0.36	0.73		0.26	NO	0.23
βArg	0.44	0.82		0.62		0.28	0.08	0.28
βPro[c]			0.30–0.50	0.67				
δLys	0.96	1.40	1.10	1.30	0.20	0.34	0.06	0.26
γPro[b]	0.72	1.20	0.50	0.98 or 0.84	0.06	0.26		0.34
γArg		1.10		1.30		0.30		0.20
γPro[c]				0.98 or 0.84				
γLys	0.54	0.94	0.52	0.98	0.12	0.28	0.10	0.36
γThr	1.50	2.90	1.60	1.60	0.66	1.40	0.54	1.00

[a] Braces indicate residues giving rise to closely spaced peaks whose T_1 values cannot be individually determined. (Reprinted from Blumenstein *et al.*[2] with permission. Copyright 1979, American Chemical Society.)

[b] Designates Pro[3] of tuftsin and corresponding Pro[4] of inhibitor.

[c] Designates Pro[3] of inhibitor.

[d] NO, not observable.

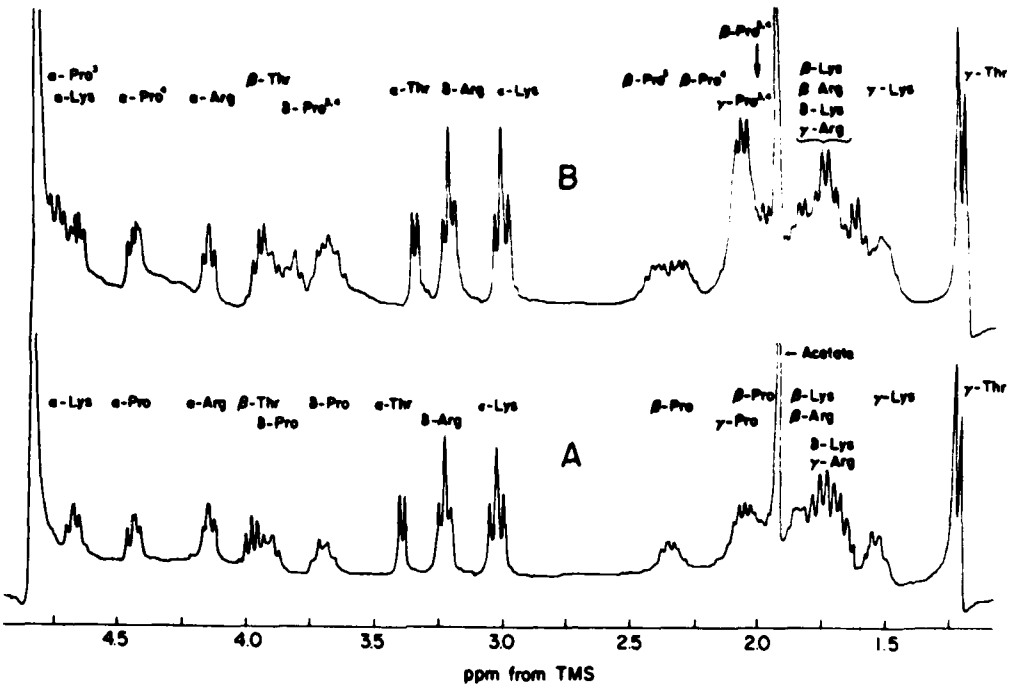

FIGURE 5. ^1H spectra at 270 MHz of tuftsin (A) and tuftsin inhibitor (B) at 0.01 *M* in D_2O, "pD" (direct meter reading) 7.5, 28°C. Number of pulses, 20; repetition rate, 3.2 sec. Reprinted from Blumenstein *et al.*[2] with permission. Copyright 1979, American Chemical Society.

constants ($J_{\alpha CH\text{-}NH}$) of the amide protons of the two peptides are listed in TABLE 3. The chemical shift of the Arg NH of tuftsin in Me_2SO is upfield of the shift observed in H_2O, and in addition, the shift in Me_2SO is independent of temperature. These observations are often indicative of a proton that is shielded from solvent and is likely hydrogen bonded.[16] The corresponding Arg NH proton in the inhibitor does not exhibit this solvent-shielded behavior, nor does the Lys NH proton of either tuftsin or the inhibitor. In water, the temperature dependence of the chemical shift of the Arg NH of tuftsin is identical to that of the Arg NH of the inhibitor, with both protons being solvent exposed.

Redfield and coworkers have developed a technique, known as saturation transfer,[17] which can be used to determine the exchange rate with solvent of exchangeable

TABLE 3. Coupling Constants (J), Chemical Shifts (Δ), and Temperature Dependence of Chemical Shifts (δΔ/°C) for Amide Protons of Tuftsin and Tuftsin Inhibitor[a]

	Lys, Me_2SO			Arg, Me_2SO			Arg, H_2O		
	J	Δ	δΔ/°C	J	Δ	δΔ/°C	J	Δ	δΔ/°C
Tuftsin	7.5	8.4	4	6.0	7.4	0	7.0	8.0	8
Inhibitor	7.5	8.4	4	6.0	7.7	4	6.5	7.8	8

[a]Coupling constants are in hertz (SD ± 0.3), chemical shifts are in parts per million from Me_4Si (±0.1), and temperature dependence (X 10^3) is in parts per million/°C (±1). Spectra were taken at 270 MHz, and chemical shifts are at 23°C. (Reprinted from Blumenstein *et al.*[2] with permission. Copyright 1979, American Chemical Society.)

protons. We have applied this technique to tuftsin and tuftsin inhibitor in Me_2SO-5% H_2O (FIG. 6), and we observe much greater saturation transfer for the Lys amide NH in tuftsin than for this proton in the inhibitor. This implies that in tuftsin this proton is exchanging with solvent at a much faster rate than occurs with the inhibitor.

DISCUSSION

Conformation in Water

Our studies do not give any indication for a particular favored conformation for either tuftsin, Thr-Lys-Pro-Arg, or the tuftsin inhibitor, Thr-Lys-Pro-Pro-Arg, in

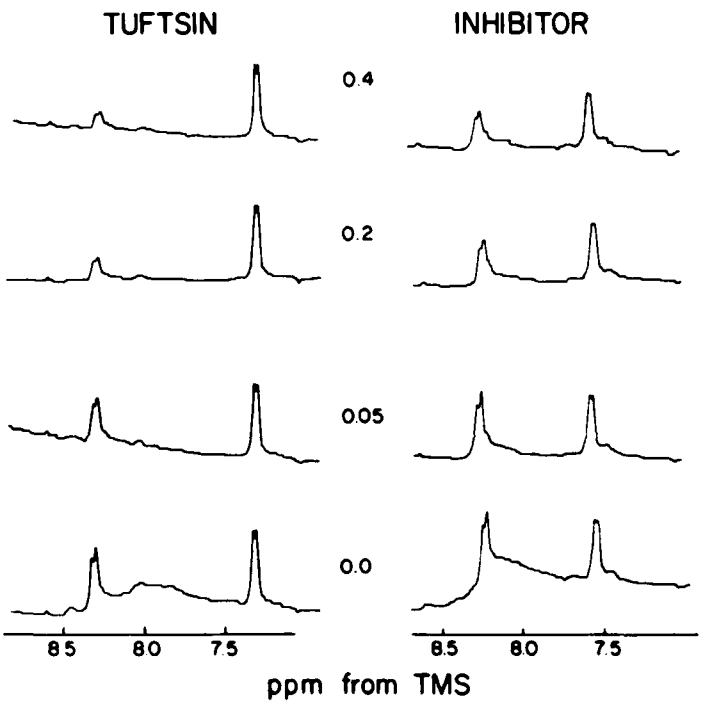

FIGURE 6. Saturation transfer experiment involving the amide NH protons and the water resonance in solutions of tuftsin and tuftsin inhibitor (0.02 *M*) in Me_2SO-d_6-5% H_2O, pH 7, 27°C. The ^1H resonance due to water was specifically irradiated for varying amounts of time indicated in the center of the figure (times in seconds). Immediately thereafter, a normal observing pulse was applied. For each spectrum, the sequence was repeated 50 times, with a 2.7-sec recycle time. Reprinted from Blumenstein *et al.*[4] with permission. Copyright 1979, American Chemical Society.

aqueous solution. Furthermore, the results eliminate many structures that have been previously proposed. Our ^{13}C pH titrations give no evidence for intramolecular interactions involving any of the molecules' charged groups. Titration of each of these groups affects the chemical shifts only of carbons within a few bonds of the group being titrated. If either the interaction between the carboxyl group of Arg and the ϵ-amino groups of Lys, as proposed by Nikiforovich[7] or the interaction between the Arg and the α-amino group of Thr, as proposed by Siemion and coworkers[1] were present, long-range titration effects would be observed. These interactions would also result in

lowered pKa values for the Arg carboxyl group and raised pKa's for the respective amino groups, while in fact the pKa values of these groups are characteristic of those observed in random coil peptides.[12] Further evidence against each of these models comes from our T_1 results. The NT_1 of the carbons of the Lys side chain increase as one goes from the α to the β carbon, characteristic of a Lys possessing segmental mobility.[18] In the model of Nikiforovich,[7] the Lys side chain would be relatively immobile, and similar NT_1 values for all carbons would be expected. In the β-turn of Siemion,[4] all α-carbons should have very similar T_1 values, while our data show increased T_1 values for the α-carbons of Thr and Arg relative to those of Lys and Pro (this effect is more clearly observed in tuftsin due to the overlapping signals of the α-carbons of Thr and Pro[3] of the inhibitor). The increased T_1 values for the terminal residues is again a characteristic of peptides having random conformations.[19] Our ^{13}C data also argue against the hairpin model,[8] which was derived from conformational energy calculations. In a conformation of this type, which is essentially a γ turn with proline in position i + 1, the β carbon of Pro would show a significant upfield shift,[20] while in tuftsin this upfield shift is not observed.

We believe the evidence for and against the β-turn model deserves further scrutiny, since Siemion and coworkers have argued extensively in favor of this model.[1,4,6] Their arguments are based on three lines of evidence: (1) optical, that is IR and cd, spectroscopy; (2) NMR spectroscopy; and (3) activity of certain analogues.

The IR measurements[6] were taken in nonaqueous media, and involved analogues of tuftsin in which all of the charged groups were blocked. These studies have little relevance to the structure of tuftsin in water. Circular dichroism measurements of tuftsin and various analogues have been performed in water.[4,5] The cd spectra have been found to be pH dependent, and both the pH dependence, as well as the spectral characteristics,[4] have been interpreted[1] in favor of a β-turn structure. While extensive studies of the influence of β turns on cd spectra have been undertaken,[21] it has not been possible to establish a definite basis for the prediction of β turns based solely on cd measurements. In particular, it has been pointed out[22] that in numerous linear peptides cd spectra led to the postulation that β-turns were present in these peptides, but that X-ray and NMR results gave no evidence for the presence of β-turns.

From ^{13}C NMR experiments on tuftsin, Siemion *et al.*[4] determined values of $-50°$ for the angle ψ of Pro and 0° or 120° for ψ of Lys. They have no information of the angle ϕ of Lys, which is also necessary to establish the existence of a β-turn. If one chooses 0° for the Lys value, they claim that these data support a type III β-turn structure.[23] Analysis of known type III β-turns indicates a value for ψ of residue i + 2 of $-30° \pm 19°$,[22] and the value 0° for Lys in tuftsin is thus outside the observed range for type III β-turns. Further confusing the issue is the assertion[1] that the Pro residue in tuftsin is in a conformation similar to that of the Pro residues in gramicidin S. In the latter peptide, Pro is in position i + 2 of a type II' β-turn. In this type of turn, position "i" must be occupied by either glycine or a D amino acid, so such a structure, which differs significantly from a type III β-turn, is not possible in tuftsin.

Our ^{13}C NMR evidence against a β-turn structure for tuftsin in water was mentioned previously. The temperature-dependent chemical shift of the Arg NH proton (TABLE 3) is further strong evidence against such a structure. If this proton participated in a hydrogen bond, its chemical shift would change much less as a function of temperature than is observed here (see our discussion below concerning the structure of tuftsin in Me_2SO). In water, our data indicate that this proton is fully exposed to solvent. Thus, the NMR evidence of Siemion and coworkers[4] does not show that a β-turn does exist in tuftsin, while our NMR evidence shows that this structure does not exist.

Finally, since D-Lys^2-tuftsin is thought to possess a greater amount of β-turn

structure than tuftsin,[4] and this analogue is active, possessing 10–20% of the phagocytic activity of tuftsin,[1] it was concluded that the β-turn structure is important for activity.[1,4] If the β-turn structure were crucial, then an analogue possessing a higher degree of this structure would be more active than tuftsin, not less. The lower activity for this analogue thus argues against the importance of the β-turn for tuftsin activity. We must, however, point out that the structure postulated for D-Lys[2]-tuftsin[4] is that of a type II′ β-turn (this structure is possible in this analogue; see above) rather than the type III turn claimed for tuftsin. Since these two structures are very different, it is not clear that any conformational conclusions concerning tuftsin can be made from studies of the analogue.

Extensive compilations of β-turns that occur in proteins have appeared.[25,22] These compilations, including several hundred β-turns, show that in not one case does a *trans* proline residue occur in position of i + 2 of a β-turn if position i + 1 is occupied by an L-amino acid. Based on this fact, the occurrence of a β-turn in tuftsin would be exceedingly unlikely, and experimental data summarized above indeed show that this structure is not present.

Conformation in Me_2SO

In Me_2SO, unlike in water, there are conformational differences between tuftsin and the pentapeptide inhibitor. These differences are manifested in the ^{13}C and ^{1}H NMR spectra. In the ^{13}C spectra, several resonances have different chemical shifts in the two compounds (TABLE 1), although the spectra are still very similar. A more dramatic difference is observed in the temperature dependence of the chemical shift of the Arg NH proton (TABLE 3). In tuftsin, the shift of this proton is independent of temperature, indicating a solvent-shielded environment, while in the inhibitor, the temperature-dependent shift indicates that the NH proton is exposed to solvent. A final difference is observed in the saturated transfer experiments (FIG. 6). The Lys NH of tuftsin undergoes exchange with water (present in 5% concentration) at a much more rapid rate than does the Lys NH of the inhibitor. The more rapid exchange rate observed with tuftsin could be due to an electron-withdrawing effect that would be observed if the carbonyl of the adjacent Thr were involved in a hydrogen bond.

The particular conformation adopted by tuftsin in Me_2SO cannot yet be determined, though the structure certainly seems to be more ordered than that present in water. Both the solvent-shielded nature of the Arg NH, and the rapid exchange of the Lys NH are consistent with a β-turn structure. Other evidence, however, argues against this structure. As mentioned above, a β-turn structure with *trans* proline in position i + 2, and an L amino acid in position i + 1 has never been observed. Also, the measured $J_{NH\text{-}CH}$ for Lys is much larger than that observed in β-turn structures,[26] and indicates an angle ϕ which is outside the range observed in a large number of β-turns.[22] While we doubt the existence of a tuftsin β-turn in Me_2SO, the evidence against this structure is not as overwhelming as is the evidence in water. Further experiments, including spatially sensitive two-dimensional NMR techniques[27] should be useful in defining the conformation in Me_2SO. In contrast to tuftsin, the pentapeptide inhibitor appears to be rather disordered in Me_2SO just as it is in water.

SUMMARY

Our NMR results indicate that neither tuftsin Thr-Lys-Pro-Arg, nor the pentapeptide inhibitor of tuftsin, Thr-Lys-Pro-Pro-Arg, possesses an ordered structure in

aqueous solution. We can rule out the existence of many previously proposed conformations, including that of a β-turn. In Me_2SO, tuftsin, but not the inhibitor, appears to possess a preferred conformation, but the details of that conformation have yet to be worked out.

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Conformational Investigations in the Tuftsin Group

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In our recent review,[1] the problem of the biologically active conformation of tuftsin was discussed. Existing models of this conformation[2-4] all suggest a folding of the molecule. The type of folding is different, however, with every proposition. The recent results of the investigations of the activity of some tuftsin analogues make each of the proposed models questionable. A distinct activity of cyclo-tuftsin, synthesized by Chippens,[5] contradicts our β-bend model. On the other hand, the inhibitory potency of Glu^2-tuftsin reported by Najjar,[6] and also the very high phagocytosis-stimulating activity found by Bar-Shavit *et al.*[7] for NH_2-terminal substance P tetrapeptide Arg-Pro-Lys-Pro, argue against Nikiforovich-Chippens's model because in both these tetrapeptides, the structure suggested by Nikiforovich is not likely.

Thus, the question of the conformation of biologically active tuftsin remains open. We do not know if the tuftsin receptor recognizes the conformation of the main chain of peptide, or the precise amino acid sequence. There exist no data concerning the contact areas responsible for peptide-receptor interaction. There is, however, much evidence on the solution conformation of tuftsin analogues. Our attempt is to provide a review of this evidence, and especially the data obtained in our laboratory.

Our own investigations of this subject were connected mainly with two problems:

(1) the conformational role of proline residue in the tuftsin molecule; and
(2) the role of ionic interactions between the charged terminal groups in the stabilization of definite tetrapeptide conformers.

The presence of the Pro residue in oligopeptides evokes the phenomenon of *cis-trans* isomerism in the peptide chain. The best proof of the existence of such an isomerism is ^{13}C-NMR spectroscopy. By means of this method, the presence of the *cis* form was established for thyrotropin-releasing hormone,[8] for angiotensin II[9] and even for a peptide as large as corticotropin.[10] Bleich *et al.*[11] have recently postulated that the *cis* form of the His-Pro bond in angiotensin II may be required for maximal binding or for its biological activity at the cellular receptor.

For the tetrapeptides Thr-Lys-Pro-Arg, Thr-Lys-Pro-Ala, and Thr-Lys-Pro-Lys, there is no evidence[12] for the presence of the *cis* form in conformational equilibrium in water (see TABLE 1). The resonances of C^β and C^γ of Pro appear in the regions typical for *trans* X-Pro bond.[13] That means that the participation of *cis* isomer of tuftsin in the binding of the peptide with receptor is rather improbable. It is also interesting that the character of the amino acid residue in position 4 of the peptide does not influence the *cis-trans* isomerism. The change of configuration of the Pro residue, however, leads to change in *cis-trans* equilibrium; for D-Pro^3-tuftsin, there appear equal amounts of *cis* and *trans* isomers of the peptide in equilibrium.

A few years ago, Wüthrich and Grathwohl[14] postulated that the appearance of *cis*-Pro isomer can serve as the measure of conformational lability of the peptides. The

TABLE 1. The Chemical Shifts of C^{β} and C^{γ} Atoms of Pro in Tuftsin and Some of Its Analogues[a]

Resonance Assignment	Thr-Lys-Pro-Arg	Thr-Lys-Pro-Ala	Thr-Lys-Pro-Lys	Thr-Lys-D-Pro-Arg
				32.6 (*cis*)
C^{β}_{Pro}	30.2	30.0	30.1	30.6
C^{γ}_{Pro}	25.5	25.4	25.4	25.1
				22.5 (*cis*)

[a]In D_2O at pD 6.4, temp. 20°C.

absence of such an isomer may be interpreted as proof that some definite conformations of the peptide chain are preferable. From this point of view, the conformation of tuftsin could be considered quite stable; the introduction of D-Pro residue into the peptide chain destabilizes its conformation to a great extent.

In the case of a more simple model of tetrapeptides composed from three Ala and one Pro residue, the tendency for a *cis*-isomer formation is much more significant.[15] The amount of the *cis* form increases slightly with increasing pD (TABLE 2); for the zwitterionic form of Ala-Pro-Ala_2 the minimal amount of *cis*-isomer appears in the solution, which suggests that the interaction between the charged terminal groups in the peptide favors a *trans*-configuration of the Ala^1-Pro^2 amide bond. These observations suggest that the absence of *cis*-isomer in the case of tuftsin may be due to the specific amino acid sequence of this peptide.

Several years ago, we developed[15-16] a simple method of conformational angle ψ determination for amino acid residues in a peptide chain. The method was based on the analysis of proton-decoupled ^{13}C-NMR spectra. We have demonstrated that ^{13}C-NMR chemical shifts of C^{β} atoms of individual amino acid residues in peptides change in parallel with the absolute value of dihedral angle θ of C^{β}-C^{α}-C'-O moiety. This angle is connected with the ψ angle in the relation $\psi = \theta + 60°$. In the beginning, we applied this method for the estimation of the ψ angle of Pro residues only.[17,18] The general application of this method is enabled by the hydantoin scale, which was introduced by us in 1978.[16,19] Because of their identity for every amino acid conformation ($\phi = \psi = 0°$), hydantoins could be used as standards for the comparison of conformation of different amino acid residues. For this purpose, we use $\Delta\delta_h$ coefficients, $\Delta\delta_h = \delta C^{\beta}_p - \delta C^{\beta}_h$, where δC^{β}_p is the chemical shift of the precise amino acid residue in the peptide, and δC^{β}_h is the chemical shift of the C^{β} atom in the corresponding hydantoin. Our results show that the residues with the same or similar values of $\Delta\delta_h$ coefficients possess the similar conformation (similar ψ angles). Based on this approach, the ψ angle for Pro^3 in tuftsin was found to be near $-50°$,[12] that is, very close to the value found for Pro in gramicidin S.[20] As the proline ϕ angle must be close to $-60°$, the complete data for the Pro residue in tuftsin were established.

TABLE 2. The Amounts of *cis* Form in Model Tetrapeptides Composed from Ala and Pro Residues[a]

Tetrapeptide	Percent of *cis* Form for		
	Cationic Form	Zwitterionic Form	Anionic Form
Ala-Pro-Ala_2	17	9	19
Ala_2-Pro-Ala	9	15–18	11
Ala_3-Pro	12	21	19

[a]In D_2O, temp. 33–37°C. See Siemion *et al.*[15]

The comparison of $\Delta\delta_h$ values of the residues that occupy the same positions in the peptide chains may serve as a simple method of establishing the conformational similarity of compared peptides. Such comparison performed with some tuftsin analogues (see TABLE 3) shows that the character of the amino acid residue in position 4 of the chain has little influence on the conformation of the Pro^3 residue. The substitution of Pro^3 by another amino acid distinctly diminishes the value of the $\Delta\delta_h$ coefficient. That means a corresponding diminishing of the θ angle. The closest conformational similarity with tuftsin shows Thr^3-tuftsin. We explained[21] this phenomenon by the existence of hydrogen bonding between the Thr-hydroxyl group and the Lys^2-Thr^3-amide bond. In this way, the spatial structure, which is very similar to that created by Pro, may be formed. Introduction of Val into position 3 of the chain distinctly increases the population of conformers with small θ angles. The introduction of Ala causes still greater changes. In the last case, in conformational equilibrium, a notable amount of conformers in which the Ala-carbonyl group eclipses the Ala-methyl substituent is present.

To better understand the role of the Pro residue in the creation of the tetrapeptide

TABLE 3. The $\Delta\delta_h$ Coefficients for C^β Resonances of Residues 2 and 3 of the Peptide Chains in the Series of Tuftsin Analogues

Peptide	$\Delta\delta_h$ for Residue (in ppm)	
	2	3
Thr-Lys-Pro-Arg	1.90	3.55
Thr-Lys-Pro-Ala	2.10	3.55
Thr-Lys-Pro-Lys	2.00	3.45
Thr-Lys-Thr-Arg	2.40	2.07
Thr-Ala-Val-Arg	0.37	1.29
Thr-Lys-Ala-Arg	2.60	0.37

conformation, we investigated by ^{13}C-NMR and CD spectroscopy the following model compounds:

Ala-Ala-Ala-Ala (I) Pro-Ala-Ala-Ala (II)
Ala-Pro-Ala-Ala (III) Ala-Ala-Pro-Ala (IV)
Ala-Ala-Ala-Pro (V)

^{13}C-NMR measurements were performed in D_2O, at pD 1.0, 4.2, and 12.5, that is, for the cationic, zwitterionic, and anionic forms of each peptide. We found (see TABLE 4) that independently of pD, the C^β_{Ala} resonance of the residue preceding Pro is shifted, as compared with the position of correspondent resonance of peptide I, by about 0.9–1.5 ppm to the higher field. In the case of peptide III, the shielding effect exerted by Pro is combined with deshielding caused by the deprotonation of the terminal Ala^1 residue. The shielding effect of Pro diminishes slightly when pD increases. For peptides IV and V, Ala preceding Pro has a negative $\Delta\delta_h$ coefficient.[15] This corresponds to such a residual conformation in which the Ala-carbonyl group eclipses the Ala side chain. According to the values of $\Delta\delta_h$ coefficients, Pro-residues in peptides III and IV possess very similar average conformation. It corresponds to *cis'*-Pro (θ about $-100°$, ψ of about $-40°$) rather than with the *trans'*-Pro conformation (θ of about 60°, ψ of about 120°). Thus, proline exerts a steric influence on the preceding alanine, favoring its conformation with small θ angles. We must note that this effect had earlier been observed not only for Ala,[22] but also for the Phe[23] residue. It is possible that such an influence may also be important for other amino acid residues.

TABLE 4. Chemical Shifts of Consecutive Ala Residues in Tetrapeptides I–V[a]

pD	Ala-Ala-Ala-Ala				Pro-Ala-Ala-Ala			Ala-Pro-Ala-Ala			Ala-Ala-Pro-Ala			Ala-Ala-Ala-Pro		
~ 1.0	17.32	17.32	17.32	16.91	17.32	17.32	16.93	15.84	17.21	16.98	17.32	16.01	16.87	17.32	17.32	15.96
~ 4.2	17.34	17.34	17.34	18.35	17.47	17.47	18.45	15.84	17.26	18.29	17.32	16.13	18.12	17.32	17.32	16.24
~12.5	20.79	17.44	17.44	18.46	17.43 17.66		18.40	19.88	17.38	18.46	20.74	16.24	18.39	20.79	17.44	16.30

[a] D_2O, ppm in relation to TMS. The resonances of Ala preceding Pro are underlined.

The resonance positions of individual carbon atoms in investigated series of tetrapeptides, except those in NH_2- and COOH-terminal residues, depend little on pD. It is remarkable, however, that $\Delta\delta_h$ coefficients of C^{β}_{Ala} atoms increase systematically upon transition to basic pD, demonstrating some decrease of the population of conformers with small θ_{Ala} angles. These changes are more distinctly visible in CD spectra of these tetrapeptides.[24]

As far as the chiroptical properties are concerned, the investigated tetrapeptides can be divided into two groups: the first composed of the peptides I, II, and IV, and the second of III and V. At low pH, the CD spectra of I, II, and IV are close to those of the peptide in random conformation. The differential spectra[24] (where the CD spectrum of I was treated as the reference) show that in the solutions of III and V, the distinct amount of β-bend conformers is present in conformational equilibrium. (For a discussion of chiroptical properties of β-bends, see Lisowski *et al.*[24])Thus, the presence of Pro in position 2 or 4 of a tetrapeptide encourages the β-bend formation even in the acid pH range.

The stimulation of β-bend formation by the Pro-residue that occupies position 2 of the tetrapeptide is a well-known phenomenon. A similar conformational influence exerted by Pro in position 4 of the chain was, however, not expected. In our opinion, the effect may be explained by the steric influence of Pro on the preceding Ala residue. When the methyl group of Ala^3 eclipses its carbonyl group, the angle ψ^3_{Ala} must be close to that typical of the i + 2 residue in the β-bend. The analogical interaction in peptide IV favors the defolding of the peptide chain, thus orienting its ends in opposite directions. Hence, the conformational differences observed in acidic medium for II and IV on one side, and III and V on the other, could be explained by the steric influence of the Pro residue, which favors in the first case defolding, and in the second case folding of the peptide.

In neutral and basic solutions, the amounts of β-bend conformations increase, independently of the position of Pro in the chain. In every case, the differential CD spectrum, obtained by subtracting $[\theta]_{\lambda}$ at pH 1.8 from $[\theta]_{\lambda}$ at pH 10.5, is close to the CD curve of the β-bend conformation. The effects depend on the ionization of the terminal carboxylic group rather than on the interaction of the terminal charged groups in the zwitterionic form of the peptide, because at the basic pH range, it is more distinct than in neutral solution. The amplitude of the effect is greater for peptides II and IV than for III and V.[24]

We must note that very similar changes in CD spectra, evoked by pH increase, were observed by us[12] for tuftsin analogues. We investigated from this point of view the following compounds:

Thr-Lys-Pro-Ala	D-Thr^1-tuftsin
Thr-Lys-Ala-Ala	D-Lys^2-tuftsin
Thr-Ala-Val-Arg	D-Pro^3-tuftsin
	D-Arg^4-tuftsin

One of the aims of this investigation was to check the influence on tuftsin conformation exerted by the change of configuration in the precise position of the peptide chain.

The CD spectra of Thr-Lys-Pro-Ala, Thr-Lys-Ala-Ala, and Thr-Ala-Val-Arg change when pH increases in a way very similar to that observed for tuftsin itself. The comparison of differential CD spectra of these compounds with those obtained for tetrapeptides composed of alanine and proline, suggests that here also the changes in CD could be interpreted as a demonstration of increasing amounts of β-bend conformations in the conformational equilibria.

The change of configuration in positions 1 and 4 of the tuftsin peptide chain does

not affect to a great extent the conformational equilibrium in solution as compared with tuftsin. The distinct effects, however, accompanied the change of configuration of the residues in position 2 or 3. Thus, residues 2 and 3 seem to be more important for the preservation of the tuftsin conformation than residues 1 and 4. As discussed above, in the case of D-Pro^3-tuftsin, a large amount of *cis*-isomer of the peptide appears in solution. It is interesting, however, that the CD spectrum of D-Lys^2-tuftsin in neutral solution resembles that of β-bend of type II'.

From these experiments, the conclusion could be drawn that tuftsin-like tetrapeptides possess a distinct tendency for β-bend formation in neutral water solutions. Based on our ^{13}C-NMR and CD data, we have concluded that in the case of tuftsin, the bend may be of type III. Still, these conclusions are not directly linked with the data on biological activity of tuftsin analogues, that is, a very potent[7] tetrapeptide with tuftsin-like activity, Arg-Pro-Lys-Pro, could easily form the β-bend of type I. There are in this tetrapeptide, two Pro residues in positions 2 and 4. Both these residues are likely to stimulate cooperatively the bend formation. The tetrapeptide Pro-Lys-Pro-Arg, a very active tuftsin analogue[5] which is the retrosequence of the above peptide, may also form a bend in neutral solution, but the spatial orientation of Pro residues should in this case be very different from the one mentioned above. For retrotuftsin (Arg-Pro-Lys-Thr), the contradictory data could be found in the literature; according to Hisatsune *et al.*,[25] it is inactive, but according to Yasumura *et al.*,[26] it is as potent as tuftsin itself. For the tetrapeptide Thr-Pro-Lys-Ala, we have found[27] no activity. The peptide chain conformation, however, in Arg-Pro-Lys-Pro, Arg-Pro-Lys-Thr, and Thr-Pro-Lys-aAla could be expected to be of a similar type.

We also investigated the folding tendency in the tuftsin group by IR technique.[28,29] The measurements were performed in organic solvents using fully protected peptides. Therefore, direct comparison of the data obtained by this technique with those from NMR and CD is rather risky. I would like to give here only some general conclusions from this set of experiments, and to concentrate the attention on some inconsistencies between IR on the one hand, and NMR and CD data, on the other. The aim of the IR investigations was to find answers to the following questions:

(1) Is the stabilization of a given peptide conformation indicated by IR?
(2) To what extent does tuftsin tend toward intramolecular hydrogen bond formation?
(3) What is the mode of the change in the folding and in the intramolecular hydrogen bond formation after a definite residue in tuftsin has been exchanged by another residue, or what is the influence of configurational changes on these properties of the peptide?

The presence of bend conformations was established by the appearance in the spectra of the characteristic absorption bands at 1625–1640 cm^{-1} and 1680–1685 cm^{-1}.[30] The tendency of intramolecular hydrogen bonding was estimated using the Shields method,[31] founded on the determination of concentration dependence of the ratio of absorption intensities I_{3310}/I_{3420}. We found that the β-bend formation is only roughly connected with intramolecular hydrogen bonding. The protected tuftsin molecule is characterized by a moderate tendency toward intramolecular hydrogen bond formation, with a simultaneous stabilization of β-bend. For Z-Thr-Pro-Lys (Z) Ala-O-Bzl (proline in position 2 of the chain), no 1630 cm^{-1} absorption band appears in the spectrum. It is visible, however, in the spectrum of Z-Thr-Lys (Z) Pro-Ala-O-Bzl (TABLE 5). This is interesting, because Pro in position 2 should stimulate the bend formation to an extent greater than Pro in position 3. The exchange of Pro^3 in tuftsin by another residue has a destabilizing effect on β-bend formation. Such an exchange,

however, leads to compounds that are more strongly intramolecularly hydrogen bonded than tuftsin itself. Therefore, it seems that Pro in position 3 of the chain limits the number of folded conformations for which intramolecular hydrogen bonding is possible. The presence of Arg and Lys (basic residues) in positions 2 and 4 of the chain favors the peptide folding, whereas this effect was not observed for the Thr^1-residue. The replacement of Thr^1 by another residue increases the tendency toward intramolecular hydrogen bonding.

The influence of configurational changes on the folding of tuftsin seems to depend on the position in which such a change is introduced. The tendency of intramolecular hydrogen bond formation was found to be greater for D-Thr^1- and D-Pro^3-tuftsin than for D-Lys^2- and D-Arg^4-tuftsin. The IR spectra of D-Arg^4-tuftsin manifest the dominance of unfolded structures in conformational equilibrium. It could be concluded therefore that the tendency for β-bend formation diminishes for protected D-Arg^4-tuftsin. This peptide in its unprotected form is, however, quite active as a phagocytosis stimulant.

TABLE 5. Absorption Bands of the 1760–1500 cm^{-1} Region in $CHCl_3$

Peptide	Positions of the Bands					
Z-Thr-Lys(Z)Pro-Arg(NO_2)O-Bzl	(1740)	1714	1680	1628		1512
Z-Thr-Lys(Z)Pro-Ala-O-Bzl	(1738)	1710	1678	1637	1602	1512
Z-Thr-Pro-Lys(Z)Ala-O-Bzl	(1750)	1710	1670		1601	1510
Z-Thr-Lys(Z)Leu-Arg(NO_2)O-Bzl	(1740)	1708	1670		1600	1515
Z-Thr-Lys(Z)Thr-Arg(NO_2)O-Bzl	(1763)	1710	1674		1600	1512

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(2) To what extent does tuftsin tend toward intramolecular hydrogen bond formation?
(3) What is the mode of the change in the folding and in the intramolecular hydrogen bond formation after a definite residue in tuftsin has been exchanged by another residue, or what is the influence of configurational changes on these properties of the peptide?

The presence of bend conformations was established by the appearance in the spectra of the characteristic absorption bands at 1625–1640 cm^{-1} and 1680–1685 cm^{-1}.[30] The tendency of intramolecular hydrogen bonding was estimated using the Shields method,[31] founded on the determination of concentration dependence of the ratio of absorption intensities I_{3310}/I_{3420}. We found that the β-bend formation is only roughly connected with intramolecular hydrogen bonding. The protected tuftsin molecule is characterized by a moderate tendency toward intramolecular hydrogen bond formation, with a simultaneous stabilization of β-bend. For Z-Thr-Pro-Lys (Z) Ala-O-Bzl (proline in position 2 of the chain), no 1630 cm^{-1}absorption band appears in the spectrum. It is visible, however, in the spectrum of Z-Thr-Lys (Z) Pro-Ala-O-Bzl (TABLE 5). This is interesting, because Pro in position 2 should stimulate the bend formation to an extent greater than Pro in position 3. The exchange of Pro^3 in tuftsin by another residue has a destabilizing effect on β-bend formation. Such an exchange,

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Effect of Tuftsin on the Migration, Chemotaxis, and Differentiation of Macrophages and Granulocytes[a]

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INTRODUCTION

The tetrapeptide tuftsin has been shown by numerous laboratories to be immunologically active in that this compound stimulates or enhances many functions of several leukocyte populations. Our laboratory has been interested in defining the properties of tuftsin *in vivo* and *in vitro*. Many of our studies have been directed toward the antineoplastic properties of this compound. We have demonstrated that tuftsin has an antitumor effect when administered *in vivo* using several murine tumors as models. The tumors used include L1210 leukemia in DBA/2 mice,[1] Cloudman S-91 melanoma also in DBA/s mice,[2,3] CH1 lymphoma in B10.$2^{a}4^{b}$p/wts mice,[4] primary Rous sarcoma in C57Bl/10Sn (B10) mice,[5] and 3-methylchloranthrene-induced sarcomas in B10 mice.[5]

Studies have been performed *in vitro* to determine which leukocyte populations are responsible for the immunologically mediated antitumor activity of tuftsin using both murine and human leukocytes as effector cells. Tuftsin enhanced the cytotoxic activity of murine natural killer cells and macrophages against Yac-1 lymphoma, L1210 leukemia, and Cloudman S-91 melanoma.[1,3,6,7] A similar enhancement of the cytotoxic response was observed when tuftsin-treated human macrophages, NK cells, or neutrophils were tested against HM39.5 melanoma and K562 leukemia cells.[7-9]

In addition to possessing antineoplastic properties, tuftsin has been shown to modify several other functions of various leukocyte populations. The original observations concerning tuftsin's ability to enhance phagocytosis was later extended by others to include an enhancement of the bactericidal activity of macrophages and granulocytes.[10,11] More recently we have begun to examine the changes that occur in leukocytes following their exposure to tuftsin. Tuftsin was shown to possess weak mitogenic activity for human peripheral blood leukocytes, murine mononuclear spleen cells, and murine bone marrow cells.[12] Preliminary experiments also indicated that tuftsin stimulated the migration of human peripheral blood mononuclear cells and granulocytes and abrogated the effects of migration inhibition factor induced by crude human tumor extracts.[13]

We have undertaken a series of investigations designed to extend our observations concerning the effects of tuftsin on leukocyte chemotaxis, migration, and differentiation. The effects of tuftsin on migration were examined using the capillary tube method

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and video microscopy. Chemotaxis was examined using blind well chambers and the effect of tuftsin on differentiation was examined by colony formation in soft agar and the cytotoxic response.

MATERIALS AND METHODS

Tuftsin

Synthetic tuftsin, synthesized by the liquid-phase method,[14] was obtained from the Takeda Chemical Co., Osaka, Japan, or synthesized in our laboratory.

Animals

Three- to 4-month-old C57BL/10Sn were raised in our laboratories. All mice were given food and water *ad libitum*.

Cell Lines

The cell lines used in this study, L1210 leukemia and $P388_{D1}$, were maintained in continuous culture *in vitro* at 37°C and grown in supplemented culture medium (SCM). The medium consisted of RPMI 1640 (Grand Island Biological Co., Grand Island, NY) supplemented with 10% fetal bovine serum (FCS, Kansas City Biological, Lenexa, KA), 20 m*M* HEPES buffer (Research Organics, Cleveland, OH), 20 m*M* glutamine (GIBCO) and 50 μg/ml gentamicin.

Preparation of Human Leukocytes

Peripheral blood was donated by healthy volunteers and heparinized. Leukocyte-enriched suspensions were obtained by dextran sedimentation (1 × g) at 37°C for 1 hr. The leukocyte suspensions were washed and resuspended in Hank's balanced salt solution (HBSS, Gibco). The cell suspensions were then separated into mononuclear and granulocyte fractions by gradient centrifugation on lymphocyte separation medium (LSM, Litton Bionetics, Kensington, MD). Mononuclear cells were collected from the gradient interface. The cell pellets (granulocytes) were collected, washed in HBSS, and the remaining red blood cells removed by water lysis. Monocytes were collected by two sequential plastic adherences (each of 1 hr duration) to a purity of greater than 95% as determined by nonspecific esterase staining.[15] Adherent monocytes were removed from the plastic dishes with 12 m*M* lidocaine.[16]

Chemotaxis Assay

Chemotaxis was performed using blind well chambers by the method described by Pike and Synderman.[17] Human mononuclear cells, monocytes and granulocytes were placed in the top compartment while tuftsin, chemotactic activated serum, or medium alone were placed in the bottom well. Following a 90-min incubation, the filters are fixed, stained, and the average number of cells per microscopic field is determined.

Migration

Migration studies were performed using both the capillary tube method described by Bull *et al.*[18] and by videomicroscopy using a system similar to that described by Willingham and Pastan.[19] Mononuclear cells in SCM (2×10^8/ml) were packed into capillary tubes, sealed at one and, centrifuged and broken at the medium-cell interface. The tubes were placed into chambers, filled with medium, and incubated with or without tuftsin for 18 hr at 37°C. Areas of migration were projected onto paper and mean area of migration calculated. The migration index (MI) was expressed as the area of migration in the presence of tuftsin divided by the area of migration with medium alone. In order to study random migration, monocytes were made to adhere to the bottom of a Dvorak chamber. The cells were then observed in the presence or absence of tuftsin by means of a Koyo TV camera attached to a Leitz Diavert inverted microscope with a Zeiss Neofluar 32X or Planapo 63X objective. The enclosed microscope stage was kept at a constant 37°C. Video output was recorded on a Panasonic time-lapse video tape recorder (NV-8030) and displayed on a Panasonic TV monitor (NV-5300), from which polaroid photographs were taken.

Cytotoxicity Assay

The cytotoxic activity of $P388_{D1}$ was determined as previously described.[6] L1210 cells were labeled with ^{51}Cr (New England Nuclear, Boston, MA), washed in HBSS and resuspended in SCM. The labeled target cells were mixed with $P388_{D1}$ cells (either tuftsin pretreated or untreated) at a 50:1 effector to target ratio and plated in microtiter plates. The plates were incubated for 18 hr at 37°C and the percentage of specific cytotoxicity calculated.

Colony-forming Assay

The effect of tuftsin on the formation of bone-marrow colonies (CFU-C) *in vitro* was examined using the culture method described by Metcalf.[20] Methylcellulose was used instead of agar in all cultures. Bone marrow cells were obtained from femurs of C57BL/10 mice and plated (5×10^4 to 1×10^5 cells/culture) in 35-mm petri dishes with or without various concentrations of tuftsin. All cultures were incubated at 37°C in a humidified 8% CO_2 atmosphere. Cultures containing lung-conditioned medium were used as positive controls.[21] All plates were scored after 8 days and clusters of 200 or more cells were considered colonies. Representive colonies were harvested with a fine-drawn pipette, cytocentrifuged onto glass slides, and stained with Giemsa.

RESULTS

Chemotaxis

The chemotactic activity of tuftsin was examined using human granulocytes, mononuclear cells, and monocytes. The results of our studies on granulocyte chemotaxis appears in TABLE 1. It can be seen that tuftsin displayed a slight chemotaxic effect at concentrations of 10^{-7}, 10^{-5}, and 10^{-3} μg/ml. Significant chemotaxis was observed with concentrations of 10^{-1} and 10^{1} μg/ml. The tetrapeptide kentsin (Thr-Pro-Arg-Lys) demonstrated no chemotactic effect at the same concentrations.

TABLE 1. The Effect of Tuftsin on Granulocyte Chemotaxis

Concentration of Tuftsin[a]	Mean No. Cells ± SE[b]
0	8 ± 1
10^{-9}	7 ± 1
10^{-7}	11 ± 2
10^{-5}	15 ± 3
10^{-3}	14 ± 2
10^{-1}	33 ± 3
10^{1}	38 ± 5

[a]μg/ml
[b]Mean number of granulocytes per 20 100X fields.

The chemotactic effect of tuftsin was also studied on peripheral blood mononuclear cells and purified monocytes. The results obtained using these two populations of cells were similar to that seen with granulocytes (TABLE 2). Tuftsin exerted minimal chemotactic activity at a concentration of 10^{-4} μg/ml. The maximal chemotactic activity of tuftsin was observed around 10^{-1} μg/ml and continued through the highest concentration tested (10^{3} μg/ml). Tuftsin never matched the strong chemotactic activity observed when lymphocyte-derived chemotactic factor was used as a positive control. As seen with granulocytes, kentsin demonstrated no chemotactic activity against either mononuclear cells or monocytes.

Migration

The migration of human mononuclear cells was significantly enhanced by concentrations of tuftsin from 10^{-2} through 10^{2} μg/ml as determined by migration from capillary tubes (TABLE 3). The MI indicated that tuftsin consistantly enhanced migration by approximately 50%. We undertook a series of studies to determine the visual changes that occur in monocytes following their exposure to tuftsin. In FIGURE 1, monocytes were observed under standard culture conditions for 30 min (a and b). Very little movement or change in morphology was observed during this time period. In

TABLE 2. The Effect of Tuftsin on Mononuclear Cell Chemotaxis

	Mean No. Cells ± SE[b]	
Concentration of Tuftsin[a]	Mononuclear Cells	Monocytes
0	8 ± 4	8 ± 3
10^{-5}	7 ± 4	ND[d]
10^{-4}	14 ± [illegible]	ND
10^{-3}	14 ± 2	ND
10^{-2}	20 ± 3	29 ± 5
10^{-1}	29 ± 4	33 ± 9
10^{1}	28 ± 5	37 ± 10
10^{2}	31 ± 5	30 ± 8
10^{3}	22 ± 4	ND
LDCF[c]	48 ± 7	56 ± 17

[a]μg/ml
[b]Mean number of mononuclear cells per 20 100X fields.
[c]Lymphocyte-derived chemotactic factor.
[d]Not determined.

TABLE 3. The Effect of Tuftsin on the Migration of Human Mononuclear Cells

	Concentration of Tuftsin[a]							
	0	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	1	10
Mean Area ± SE[b]	27.1 ± 3.4	27.3 ± 3.3	31.8 ± 0.5	33.1 ± 0.3	43.0 ± 1.1	38.1 ± 2.9	36.6 ± 2.7	40.3 ± 11.7
MI[c]	1	1.0	1.17	1.22	1.58	1.41	1.35	1.48
P value[d]	—	NS[e]	NS	NS	<0.0025	<0.05	<0.025	<0.01

[a]μg/ml
[b]Area in mm, SE-standard error.
[c]Migration index.
[d]As determined by Student's t-test.
[e]Not significant.

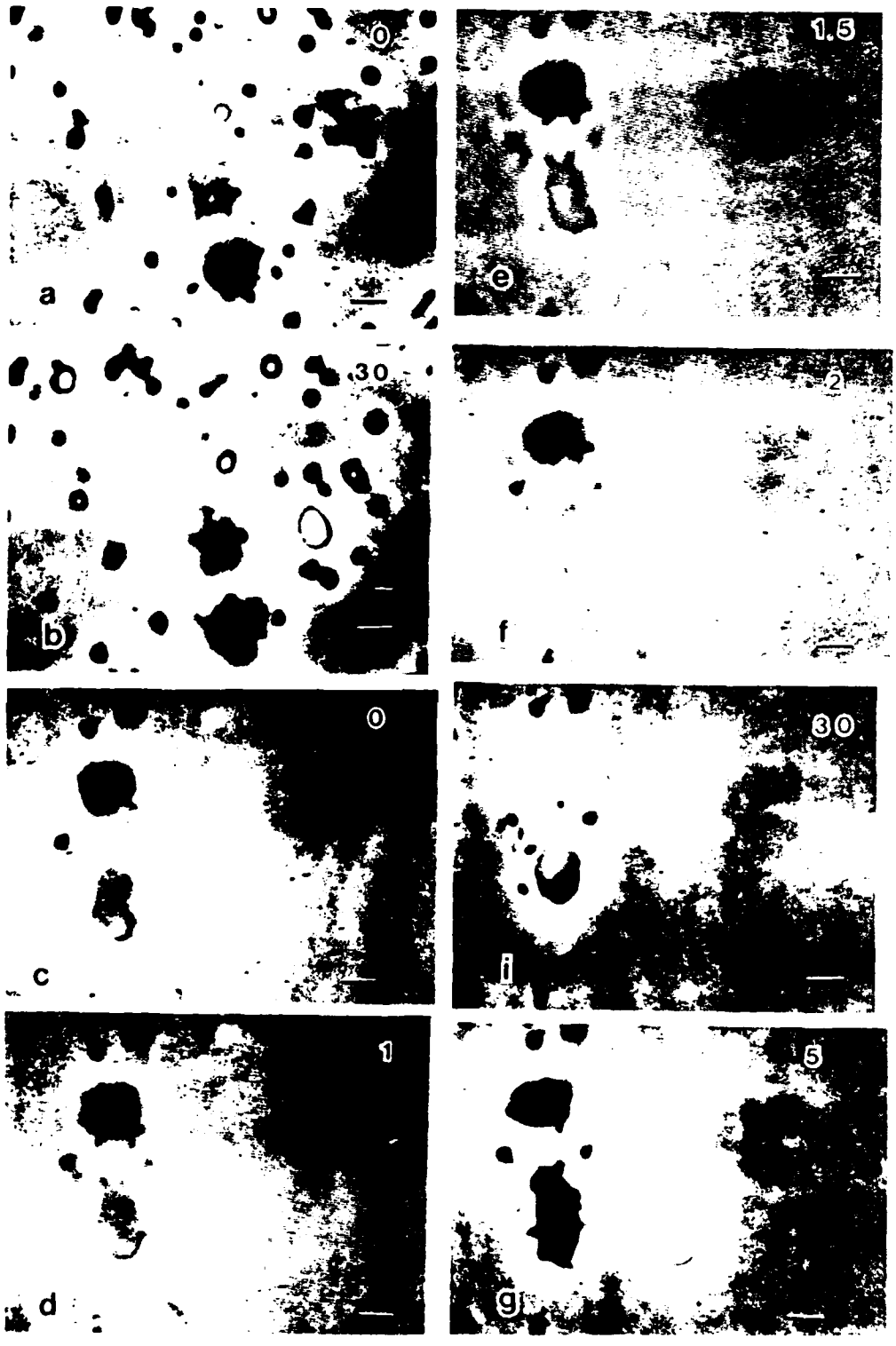

FIGURE 1. The effect of tuftsin on human monocytes in culture as determined by video microscopy. Cells were observed through a Neofluar 32X long working distance, phase-contrast lens. The final magnification was 570X. (Reduced to 60% as shown here.) Cells were observed for various times as stated in the upper right corner in minutes after the addition of 10 μg/ml of tuftsin.

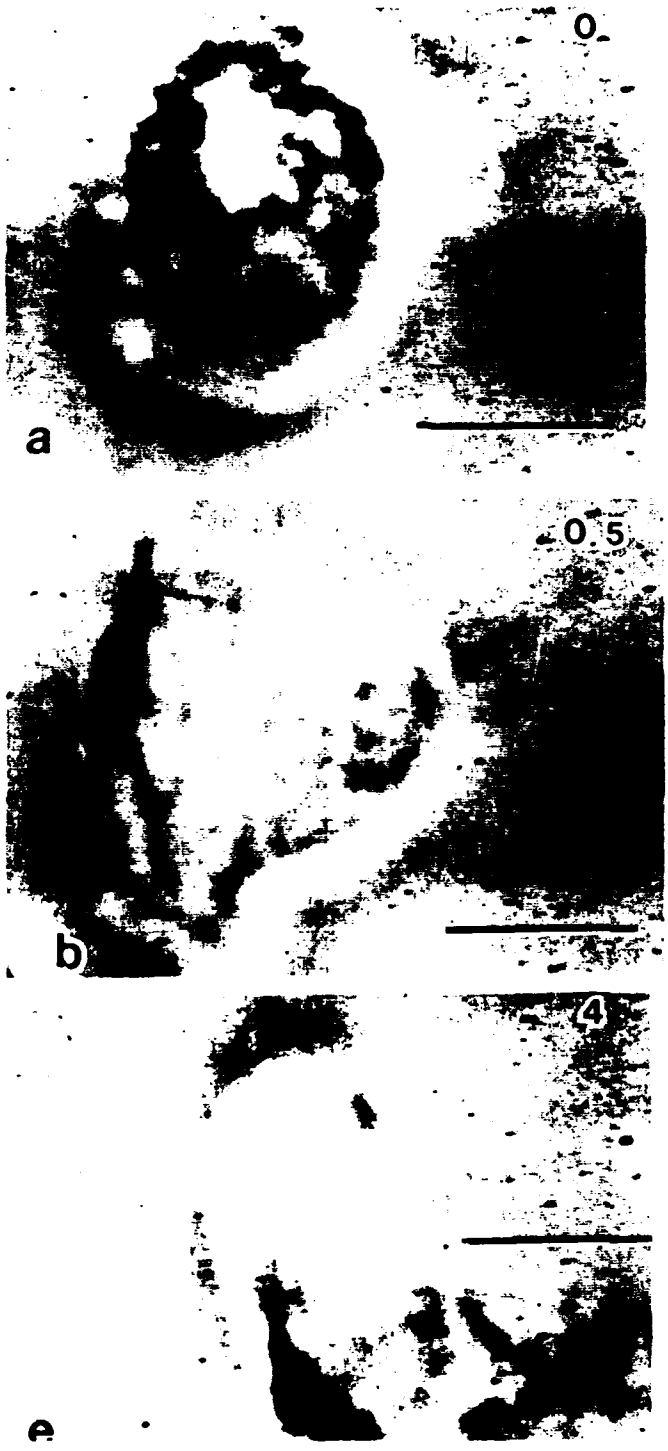

FIGURE 2. Human monocytes in the presence of 10 μg/ml of tuftsin as observed through a Planapo 63X phase-contrast lens. The numbers in the upper right hand corner represents the time in minutes following the addition of tuftsin. The bar represents 10 μm and the final magnification is 200X. (Reduced to 80% as shown here.)

contrast, monocytes in the presence of tuftsin (c-i) began to spread in less than 1 min (d). Movement could be seen by 1.5 min and continued for 5 min (g). By the end of the 30 min observation period (i), the monocytes rounded up and became loosely adherent. When the tuftsin-treated cells were observed under high magnification (FIGURE 2), morphological changes could be seen in less than 30 sec (a and b). Greatly enhanced cytoplasmic streaming and saltation occurred within seconds after tuftsin was added to the Dvorak chamber. The monocytes then became extended and began to migrate (e). The activity was maximal at a tuftsin concentration of 10 μg/ml. These cells displayed slightly less activity at tuftsin concentrations of 5 and 20 μg/ml while very little increase in activity was observed at 1 μg/ml (TABLE 4). No enhanced activity was observed in monocytes exposed to 0.01, 0.1, or 100 μg/ml of tuftsin.

Differentiation of $P388_{D1}$ and Murine Bone Marrow Cells

The ability of tuftsin to initiate differentiation in monocytes was investigated using $P388_{D1}$ cells. This cell line is composed of immature monocytes that are not cytotoxic

TABLE 4. Morophological Changes and Random Migration of Human Macrophages Induced by Tuftsin

Concentration Tuftsin (μg/ml)	Relative Change in Activity	Observations
0.01	None	No observable changes.
0.1	None	No observable changes.
1.0	±	Moderate membrane ruffling and cytoplasmic streaming.
5.0	+	Greatly enhanced membrane ruffling, cytoplasmic streaming, and saltation; enhanced migration.
10	+ +	Greatly enhanced migration, saltation, cytoplasmic streaming, and membrane ruffling.
20	+	Same as observed with 5 μg/ml.
100	None	No observable changes.

for tumor cells unless induced to differentiate. $P388_{D1}$ cells were cultured in the presence or absence of tuftsin for various periods of time after which time the rate of division, as determined by incorporation of ^{3}H-thymidine, and the cytotoxic activity for L1210 cells were determined. It can be seen in TABLE 5 that tuftsin significantly increased the incorporation of ^{3}H-thymidine at 48 hr at a concentration of 10 or 100 μg/ml and at 72 hr at a concentration of 100 μg/ml. Lower concentrations produced no effect. A cytotoxic activity of 27 and 22% were observed against L1210 targets when $P388_{D1}$ cells were cultured with 100 and 10 μg/ml of tuftsin, respectively. No cytotoxicity was observed when lower concentrations of tuftsin were used. These results are similar to those obtained with lipopolysaccharide.

Bone Marrow Colony Formation

The ability of tuftsin to enhance the differentiation of committed progenitor cells in murine bone marrow was examined using CFU-C formation in soft agar. Tuftsin

concentrations from 0.1 to 1.0 μg/culture significantly enhanced CFU-C formation (FIGURE 3). The optimal concentration (0.5 μg) displayed colony-stimulating activity equivalent to that observed with CSF. The colonies were found to contain either mononuclear or granulocytic cells.

DISCUSSION

In this study, we examined the effects of tuftsin on several events associated with monocyte and neutrophil activation. The chemotactic activity of tuftsin was examined; in addition, its effect on random migration, and differentiation were also studied. At the appropriate concentrations, tuftsin displayed chemotactic activity similar to most other commonly used chemotactic substances. These results are in disagreement with results reported by Goetzl, who observed no chemotactic activity with tuftsin.[22,23] We do not know the reason for these differences between the data from the two laboratories, but as we have previously shown, the purity of tuftsin can greatly influence its activity.[24] If tuftsin is chemotactic *in vivo*, it would have biological significance by drawing more effector cells into a site of infection (inflammation) or into a tumor.

Tuftsin's ability to enhance random migration would also be important from the biological standpoint to mobilize cells at the site where they are needed. In contrast to our data, Horsmanheimo *et al.*[25] found that while tuftsin enhanced the migration of human mononuclear cells, it did not enhance the migration of granulocytes at a concentration of 10–100 μg/ml. Goetzl[22,23] reported observing no enhancement of

TABLE 5. The Effect of Tuftsin on $P388_{D1}$ Cells

Substance Tested	Concentration μg/ml	Time Cells Harvested (hr)	^{3}H-Thymidine Incorporated (DPM × 10^4 ± SE)	P<[a]	% Cytotoxicity ± SE
None	—	48	83 ± 7.2	—	0.8 ± 3
	—	72	32 ± 3.8	—	ND[c]
	—	96	19 ± 0.8	—	ND
LPS	1	48	113 ± 9.1	0.05	34 ± 5
	1	72	56 ± 1.5	0.005	ND
	1	96	48 ± 1.4	0.0025	ND
Tuftsin	100	48	121 ± 8.9	0.05	27 ± 4
	10		1.04 ± 10.0	0.05	22 ± 3
	1		81 ± 4.1	NS[b]	3 ± 2
	0.1		9.3 ± 6.3	NS	1.4 ± 0.5
	100	72	48 ± 4.1	0.05	ND
	10		20 ± 10	NS	ND
	1		15 ± 5.8	NS	ND
	0.01		43 ± 14	NS	ND
	100	96	14 ± 2.1	NS	ND
	10		20 ± 0.3	NS	ND
	1		19 ± 2.3	NS	ND
	0.1		21 ± 5.1	NS	ND

[a]As determined by Student's t-test.
[b]Not significant.
[c]Not determined.

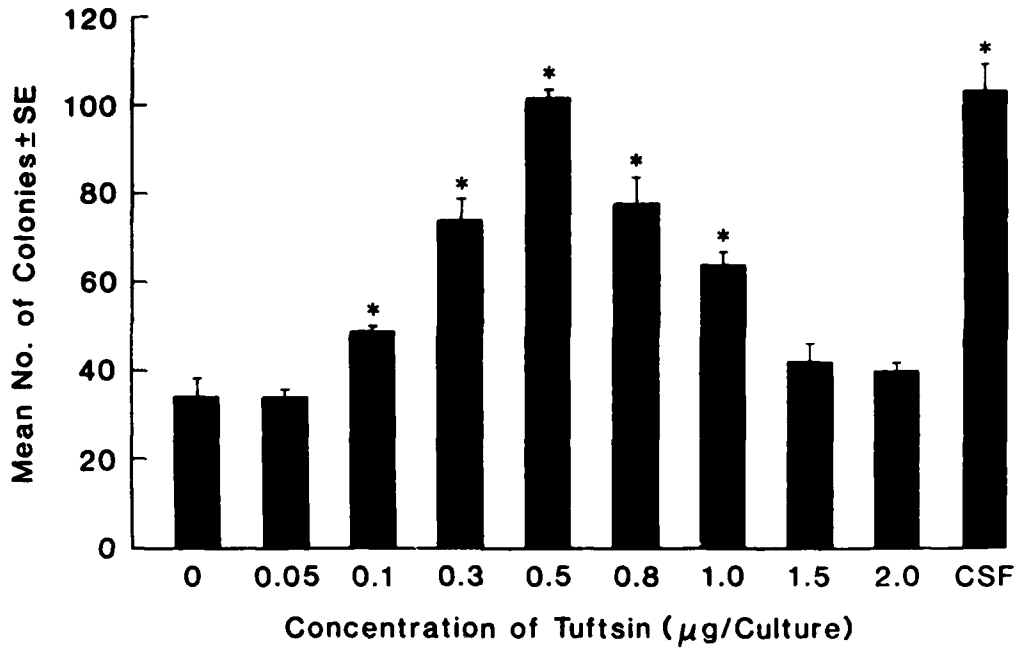

FIGURE 3. The effect of tuftsin on murine bone marrow cells cultured *in vitro*. Significance was determined by Student's t-test. The positive control consisted of bone marrow cells plus colony-stimulating factor (CSF). All cells were cultured for 8 days.

either the mononuclear or granulocyte response. Again, this discrepancy could be related to the purity of the various tuftsin preparations used.

Hematopoetic stem cells can be induced to form colonies *in vitro* that consist of cells in more mature stages of development. The stem cells detected in the assays used in our laboratory are committed to a specific pathway rather than being truly multipotential.[20] In the presence of tuftsin, the number of colonies were greatly increased. These cells appeared to be in the myelomonocytic lineage. This is not surprising since the peripheral cells of this lineage bear tuftsin receptors. We do not know if tuftsin acts directly on the stem cells or causes the macrophages, which are present in bone marrow, to secrete CFS.

In summary, the peptide tuftsin enhances or stimulates several parameters of monocytes and granulocytes associated with "activation." We have examined the effect of tuftsin on migration and chemotaxis and found it enhances both. In addition, tuftsin increases DNA synthesis in $P388_{D1}$ cells, and activates them to become cytotoxic for tumor cells. Tuftsin also greatly enhances the formation of CFU-C *in vitro* using mouse bone marrow cells.

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Enhancement of Endogenous Xenotropic Murine Retrovirus Expression by Tuftsin[a]

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INTRODUCTION

Alterations in gene expression in response to external stimuli form the basis for various states of normal and abnormal differentiation. The induction of latent virus genomes has been shown from a myriad of cell types with a variety of chemical and physical agents. With type C viruses, the virus genomes are transmitted from one generation to another as part of the host chromosome and expression can occur spontaneously with age or following chemical treatment. Chemicals that block protein synthesis[1,2] and halogenated pyrimidines[3,4] have been shown to be very efficient inducers of xenotropic virus. Spontaneous production of type C RNA viruses has been reported in mouse and rat cells transferred frequently at high densities for extended periods of time.[5-7] It has been known for some time that in rapidly dividing, DNA-synthesizing cells, the replication of mouse leukemia virus is increased.[8] In addition, superinduction of endogenous type C viruses has been demonstrated in transformed, tumorigenic subclones derived from the Balb/3T3 mouse.[9] Superinducibility appears to be associated with the loss of growth control and the transformed phenotype, presumably because these cells are less able to control normally repressed virus genetic information.

In the present report, we describe the increased expression of an endogenous xenotropic type C virus following exposure of cells to the tetrapeptide, tuftsin (Thr-Lys-Pro-Arg). Tuftsin is the active component of leukokinin, a cytophilic molecule that carries γ-globulin.[10] Treatment of phagocytic cells, granulocytes, monocytes, and macrophages with tuftsin results in phagocytosis and pinocytosis,[11-14] chemotactic migration,[13,14] bacteriocidal activity,[15] immunogenic function,[16,17] and tumoricidal and tumoristatic activity.[17,18] At present, it is believed that tuftsin-specific receptors, which confer susceptibility, are present only on phagocytic cells.[19] This

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report shows that increased expression of type C virus occurs when highly transformed fibroblasts are exposed to this tetrapeptide. Part of this work has been presented elsewhere.[20,21]

MATERIALS AND METHODS

Cell Cultures

K-Balb 19a cells cloned from a Kirsten sarcoma virus-transformed Balb/c3T3 cell line (K-Balb) were originally obtained from Dr. S. A. Aaronson (National Cancer Institute, Bethesda, MD). This clone was chosen for virus induction studies because of high levels of type C RNA virus expression following treatment with inducers.[22] This clone was used in the induction studies after having undergone long-term propagation at high cell densities (>50 subcultures), and will be referred to here as K-Balb 19a/h.[21] Fischer rat embryo (FRE) cells were obtained originally from Dr. E. Scolnick (National Cancer Institute, Bethesda, MD). BLP, a contact-sensitive Balb/c mouse embryo cell, and SLP, a contact-sensitive NIH Swiss mouse embryo cell, were kindly provided by Dr. M. Hatanaka (National Cancer Institute, Bethesda, MD); MiCl, a clone of S^+L^- mink cells, 81c, a clone of S^+L^- cat cells, and mink lung cells were kindly provided by Dr. B. Hampar (National Cancer Institute-Frederick Cancer Research Facility, Frederick, MD). All cells were grown in Eagle's minimum essential medium (EMEM) containing 10% fetal calf serum, 100 IU penicillin and 100 μg/ml streptomycin, except the mink cells, which were grown in RPMI 1640 medium. All cells were tested and found free of mycoplasmal contamination in tests performed by R. Del Giudice (Frederick Cancer Research Facility, Frederick, MD). All chemicals were from commercial sources. Tuftsin, cycloheximide, 5-iododeoxyuridine, and mitomycin C were obtained from Calbiochem, LaJolla, CA.

Induction of Virus

The assay for virus induction was a modification of the procedure described previously.[23] K-Balb 19a/h cells were plated at 5×10^5 cells/6-cm dish in EMEM containing 5% fetal calf serum and grown at 37°C for 24 to 32 hr before changing to induction medium. Induction medium consisted of EMEM containing the chemical inducer, antibiotics, 10% fetal calf serum, and 0.1 μg/ml dexamethasone to enhance virus production. After incubation in induction medium for the specified time, the drug was removed and the cells were washed three times with phosphate-buffered saline (PBS), then incubated in the dark in medium containing 20 μg/ml of mitomycin C. After 1 hr, the mitomycin C-containing medium was removed. The cells were washed three times with PBS and trypsinized. At that point, either 5×10^4 or 1×10^5 cells were plated in 6-cm dishes seeded the previous day with 1.5×10^5 FRE cells in 2 μg/ml polybrene. All focus-forming assays used FRE indicator cells unless otherwise stated. Simultaneously, 3.5×10^5 treated or untreated cells were plated in EMEM into 6-cm dishes. The next day these cells were trypsinized and counted to determine viability. Foci were counted 7 to 10 days later.

Macromolecular Synthesis

The synthesis of protein, RNA, or DNA was measured by the amounts of radioactive leucine, uridine, or thymidine, respectively, incorporated into trichloracetic

acid (TCA)-insoluble material. K-Balb 19a/h cells were treated with tuftsin for the specified time and incubated with either 2 μCi/ml L-leucine [4,5-^{3}H(N)] (New England Nuclear, Boston, MA, 5.0 Ci/mmole), 2 μCi/ml uridine [^{3}H(G)] (New England Nuclear, 3.6 Ci/mmole), or 2 μCi/ml thymidine [methyl-^{3}H] (New England Nuclear, 6.7 Ci/mmole). After 30 min, the radioactive medium was removed and the cells were washed twice with cold PBS. Each dish was washed with three, 3-ml aliquots of cold 5% TCA for 1 hr, followed by two, 15-min washes of cold 5% TCA at 3 ml each. The precipitates were dissolved with 1 ml of 0.1 *N* NaOH at 37°C for 1 hr and were neutralized with 0.1 ml of 1 *N* HCl, then were pipetted into scintillation vials, each containing 10 ml of Hydromix (Yorktown Research, S. Hackensack, NJ) and counted. Simultaneously, macromolecular synthesis was measured in untreated controls. All values were determined in triplicate.

Cellular Growth Studies

The K-Balb 19a/h cell line was plated at 5×10^4 cells per 6-cm dish in EMEM. The following day, the cells were refed on growth medium containing three concentrations of tuftsin. The cultures were refed again on day 5. The cultures were detached by trypsinization and counted by trypan blue exclusion on each of days 1 through 5 and on day 7. Three dishes in each experiment at each concentration and time point were used to determine cell growth. The cell numbers represent the mean and standard deviation as determined from the dishes in duplicate experiments.

RESULTS

Increased Virus Expression by Tuftsin

Enhancement of virus expression by tuftsin was examined and a dose-response relationship determined (FIG. 1). The maximum enhancement of virus occurred following an 18-hr incubation with tuftsin at 100 μg/ml (2 $\log_{10}$). When cells were treated with 1000 μg/ml of the tetrapeptide, a lower number of foci was observed. This was not due to cytotoxicity because, at 1000 μg/ml, the relative plating efficiency (RPE) was only 2% below the 100 μg/ml RPE level. The results indicate that the rate of enhanced virus expression was a function of increased tuftsin concentration up to an optimum, and that the reduced enhancement level beyond optimum was not a consequence of cytotoxicity.

As little as 1 hr incubation with the tetrapeptide was necessary to show enhanced virus expression (FIG. 2). Tuftsin-mediated virus enhancement remained comparable with that of cycloheximide for up to 3 hr incubation, but was approximately 25% lower after 4 hr. Similarly, enhancement of virus expression by tuftsin was comparable with that of 5-iododeoxyuridine (IUdR) for up to 2 hr incubation, inducing 42 foci compared with 57 foci for IUdR but was approximately 70% lower after 3 hr (data not shown). Therefore, the number of foci increased with an increase in the exposure time of K-Balb 19a/h cells to the tetrapeptide.

During the course of these experiments, we noted that virus release from K-Balb 19a cells was not always susceptible to tuftsin treatment, especially with cells that had been propagated for less than 50 subculture generations. On several occasions, we also noted a very low level of spontaneous virion release from the K-Balb 19a/h cells. The relationship between enhanced retrovirus expression by tuftsin and spontaneous release over an extended time period is illustrated in FIGURE 3. The level of

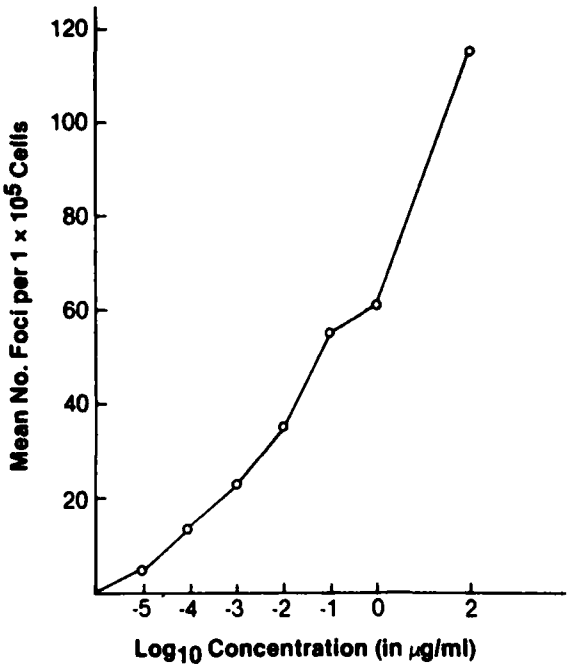
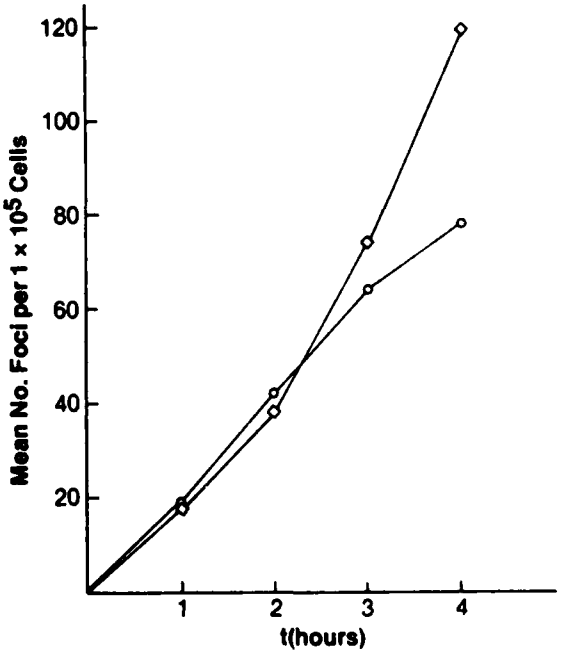


FIGURE 1. Viral expression with increasing concentrations of tuftsin. K-Balb 19a/h cells were plated at 5×10^5 cells in complete medium and were grown at 37°C for 24 hr. The cells were then induced for 18 hr in EMEM with increasing concentrations of tuftsin plus 0.1 μg/ml dexamethasone which has been shown to stimulate viral expression.[34] The cultures were then treated with 20 μg/ml mitomycin C for 1 hr, washed, trypsinized, and 1×10^5 cells were transferred to FRE monolayers that had been seeded the previous day with 1.5×10^5 cells in EMEM containing 2 μg/ml polybrene. Simultaneously, 3.5×10^5 treated or untreated cells were plated in EMEM into 6-cm dishes. The next day these cells were trypsinized and counted to determine viability; foci were counted 7 to 10 days later. Tuftsin, (O—O).

tetrapeptide-mediated expression appeared to correlate closely with the level of spontaneous release of virus.

Other clones of K-Balb cells from which virus can be activated at high frequency, following treatment with IUdR or cycloheximide, responded less well to treatment with tuftsin, as did low-passaged K-Balb 19a cells. This suggests that the increased expression of virus by K-Balb 19a/h cells after treatment with the tetrapeptide may be the property of an unusual variant selected *in vitro*, and not necessarily a general property of Balb/c mouse cells.

FIGURE 2. Tuftsin-mediated virus expression as a function of time. K-Balb 19a/h cells were plated at 5×10^5 cells in complete medium and were grown at 37°C for 24 hr. The cells were then induced for 18 hr in EMEM with increasing concentrations of tuftsin or cycloheximide plus 0.1 μg/ml dexamethasone, which has been shown to stimulate viral expression.[34] The cultures were then treated with 20 μg/ml mitomycin C for 1 hr, washed, trypzinized, and 1×10^5 cells were transferred to FRE monolayers that had been seeded the previous day with 1.5×10^5 cells in EMEM containing 2 μg/ml polybrene. Simultaneously, 3.5×10^5 treated or untreated cells were plated in EMEM into 6-cm dishes. The next day these cells were trypsinized and counted to determine viability; foci were counted 7 to 10 days later. 10 μg/ml tuftsin, (O—O); 25 μg/ml cycloheximide (◊—◊).



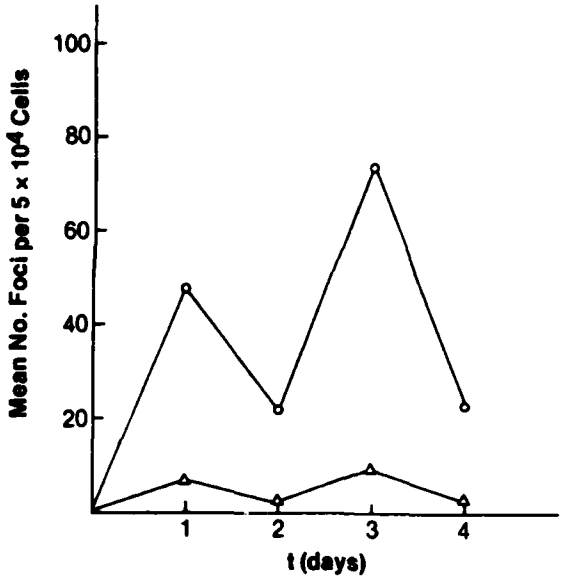


FIGURE 3. Relationship between tuftsin-mediated induction and spontaneous release of virus over a time period. K-Balb 19a/h cells were plated at 5 × 10^4 cells/6-cm dish and focus-formation assays performed as described in FIGURE 1. Cells plated on day 0 were permitted to grow without refeeding, except at the time when the medium was replaced with induction medium. Treatments were carried out for 16 hr. Control (spontaneous release) (△—△); 100 μg/ml tuftsin (○—○).

Induction by both IUdR and cycloheximide requires *de novo* cellular RNA synthesis, and their activity as inducers can be blocked by treatment with actinomycin D.[24] During activation by both classes of chemicals, viral-specific RNA accumulates in both nucleus and cytoplasm, suggesting that transcriptional derepression may occur.[25,26] Sensitivity of virus induction to α-amanitin in intact cells was shown at concentrations known to specifically inhibit transcription by RNA polymerase II, thereby providing direct support for transcriptional derepression during induction.[27] It was of interest, therefore, to test the effect of inhibiting RNA synthesis on tuftsin-mediated enhancement of virus (TABLE 1). K-Balb 19a/h cells were treated with tuftsin for 18 hr in the presence or absence of 1 m*M* actinomycin D and then assayed for focus formation. Tuftsin-mediated virus induction was inhibited 94% by treatment with actinomycin D, suggesting a requirement for *de novo* cellular RNA synthesis in tuftsin-mediated release of retrovirus from K-Balb cells.

To show that a type C virus was associated with the appearance of foci on FRE cells, neutralization experiments were carried out with goat anti-RLV gp70 serum.[28] As indicated in TABLE 2, neutralization abolished transformation of FRE cells. The data show that anti-RLV gp70 serum completely neutralized the focus-forming ability

TABLE 1. *Inhibition of Tuftsin-mediated Virus Enhancement*

Treatment[a]	Mean No. Foci/Dish	Mean Percent Inhibition	Plating Efficiency (%)
None	1	—	100
IUdR (30 μg/ml)	1543	—	77
Cycloheximide (25 μg/ml)	1105	—	63
Tuftsin (100 μg/ml)	137	—	93
Actinomycin D (1 m*M*) + IUdR	6	>99	78
Actinomycin D (1 m*M*) + Cycloheximide	7	>99	69
Actinomycin D (1 m*M*) + Tuftsin	8	94	69

[a]K-Balb 19a/h cells were treated for 18 hr with the inducers in the presence and absence of 1 m*M* actinomycin D and then assayed for focus formation as described. The mean number of foci is derived from two separate experiments, containing two dishes for each treatment.

TABLE 2. Neutralization of Virus Infectivity with Anti-RLV gp70[a]

Treatment	Percentage Inhibition at Dilutions of							Viability (%)
	None	1:10	1:20	1:40	1:80	1:160	1:320	
None	(2)[b]	NT[c]	NT	NT	NT	NT	NT	100
IUdR (30 μg/ml)	(524)	85(80)	67(172)	53(246)	10(474)	1(523)	0(554)	94
Tuftsin (100 μg/ml)	(12)	92(1)	75(3)	67(4)	75(3)	50(6)	17(10)	109

[a]Neutralization was determined with varying dilutions of anti-RLV gp70 serum after the induction period, at the time of cell overlay in the focus formation assay. K-Balb 19a/h cells were plated at 3×10^5 cells/3.5-cm dish. The focus formation assay was then performed as described. Antisera were added to the overlaid monolayers for 4 hr, which were then washed and complete EMEM was added. Nonspecific goat anti-IgG was used as a control in some experiments.

[b]Numbers in parentheses indicate the mean number of foci per 3.5-cm dish.

[c]NT, not tested.

of tuftsin-treated K-Balb cells with a 50% endpoint titer of between 1:160 and 1:320. The focus-forming ability of IUdR-mediated virus induction was neutralized with a 50% endpoint titer of 1:40. Neutralization was not a consequence of toxicity, as determined by the viability studies and appearance of the cells during the experimental procedure.

The temporal decay of viral expression after removal of tuftsin, cycloheximide, and IUdR was performed to compare the induction process further. TABLE 3 shows that viral expression induced by cycloheximide and tuftsin declined to about the same level after 24 hr, while viral expression induced by IUdR did not. As shown by others, viral expression induced by IUdR decays more slowly than that induced by cycloheximide.[29] Additional experiments using tuftsin suggest an exponential decline in viral expression. The similarity between the temporal decay of viral expression by tuftsin and cycloheximide activation appears consistent with the concept that this inducer affects a common regulatory mechanism.

Two pseudotypes of endogenous type C viruses have been identified in K-Balb cells[30] and there is evidence for partial expression of a third virus.[31] Experiments were

TABLE 3. Temporal Decay of Induced Virus

Treatment[a]	Mean No. Foci/Dish at		Percent Viability at	
	0 hr	24 hr	0 hr	24 hr
None	5	4	100	100
IUdR (30μg/ml)	1600	776	95	101
Cycloheximide (25 μg/ml)	152	15	143	95
Tuftsin: 100 μg	82	16	95	105
10 μg/ml	87	6	93	113

[a]K-Balb 19a/h cells were induced for 16 hr and either assayed for virus at that time (0 hr) or refed with complete EMEM for 24 hr and then assayed for virus.

carried out to determine the host range of virus produced in response to tetrapeptide treatment (data not shown). Tuftsin-treated K-Balb cells produced transformed foci on FRE only, and not on Balb/c mouse, NIH Swiss mouse, mink, human, or cat cells. Transformed foci arising from FRE cells were cloned and serially cultured, and focus formation assays were performed using filtered supernatants on the various indicator lines. Foci appeared on the FRE, mink, human, and cat cells, but not on the Balb/c or NIH Swiss mouse cells. Thus both the virus released initially from K-Balb cells following tetrapeptide treatment, and the virus released from the FRE cells had a xenotropic host range.

Effect of Tuftsin on Biosynthesis

Most agents that induce virus alter the synthesis of macromolecules. Therefore, in an attempt to explain virus induction by tuftsin, biosynthetic analyses were made through isotope incorporation. Tuftsin either had no effect or stimulated DNA, RNA, and protein synthesis in K-Balb 19a/h cells during short (4-hr) or long (16-hr) incubations. In all cases, the longer exposure time caused a greater increase in DNA synthesis than the shorter exposure time. A dose-response study on the effect of macromolecular synthesis by tuftsin showed no real concentration-dependent differ-

TABLE 4. Effect of Tuftsin on Biosynthesis

Treatment[a]	Percentage of Isotope Incorporation for		
	[^{3}H]thymidine	[^{3}H]uridine	[^{3}H]leucine
None	100	100	100
Tuftsin: 100 μg/ml	106	120	102
1 μg/ml	120	104	114
0.01 μg/ml	113	119	101

[a]K-Balb 19a/h cells were treated for 16 hr with tuftsin, the last 0.5 hr 2 μCi/ml of the isotope was added. Data expressed as percentage incorporation for each concentration compared to untreated control. The synthesis of protein, RNA, and DNA was measured by the amounts of radioactive leucine, uridine, and thymidine incorporated into trichloracetic acid (TCA)-insoluble material as described. All values were determined in triplicate.

ence on the rate of the isotope incorporations (TABLE 4). At the three concentrations indicated, no discernible differences were observed in protein synthesis, while there were increases in the levels of DNA and RNA synthesis. The data suggest that the basis of virus induction by this compound is different from that of previously identified inducers, such as cycloheximide and IUdR. Moreover, tuftsin may require stimulation of DNA synthesis for virus induction to occur; in this respect, tuftsin-mediated virus activation appears similar to that of immunological induction.[32]

Cellular Growth Studies

In order to determine if the increases in macromolecular synthesis were in concert with an enhanced cellular growth rate, K-Balb 19a/h cells were plated in complete growth medium for 24 hr, followed by the addition of three concentrations of tuftsin; the cultures were then counted on successive days. The histogram of FIGURE 4 indicates that the growth rate of these cells is either constant or enhanced when compared with control values, depending on the concentration of tuftsin. Tuftsin at 1.0 and 0.01 μg/ml enhanced the growth rate of these cells over time, which appears to correlate with the observed increased level of DNA synthesis.

DISCUSSION

Chemical induction of endogenous type C virus from mouse cells has provided a useful system to explore the action of tuftsin on gene expression. Genetically transmitted viral genes and transforming genes are now accepted as part of the normal genetic makeup of many organisms and as being activated by a variety of agents.[2,4,29,33] The endogenous retroviruses with their capacity to recombine with cellular genes have the ability to transfer information between cells and presumably within a cell, like bacterial insertion sequences. Activation of the retrovirus genome in response to external stimuli may be a consequence of alteration in gene expression resulting in a differentiated state. As a means of derepressing viral gene expression, tuftsin was tested as an inducer of virus activation. The present experiments show that tuftsin is able to trigger expression of endogenous type C virus from a highly transformed clone of K-Balb cells selected by continuous passage in culture at high cell densities. Our observations that tuftsin is active in a fibroblast culture system suggest that the

tetrapeptide may be involved more widely in the regulation of normal cell replication and differentiation than was previously thought. The increased expression of virus appears to be specific for the peptide sequence since Gly-Leu-Gly-Leu, Leu-Tyr-Leu, Thr-Lys-Phe, Lys-Lys-Lys-Lys, and Try-Gly-Gly-Phe-Met did not increase virus expression (data not shown). Also, tuftsin did not act synergistically with IUdR to enhance virus production, indicating a difference in mechanism to that of dexamethasone.[34] The mechanism by which this tetrapeptide alters virus expression is unclear, but could take place through peptide interaction with cell membrane receptors similar to that reported for polypeptide growth factors[35-37] or through an interaction of specific amino acid sequences with nucleic acid, since it has been shown that peptides such as Gly-His-Gly and Lys-Trp-Lys bind to single-stranded DNA regions.[38-40] A preliminary report noted that tuftsin increased the level of both virion-associated reverse transcriptase and budding virus from murine leukemia virus-shedding mouse cells.[41]

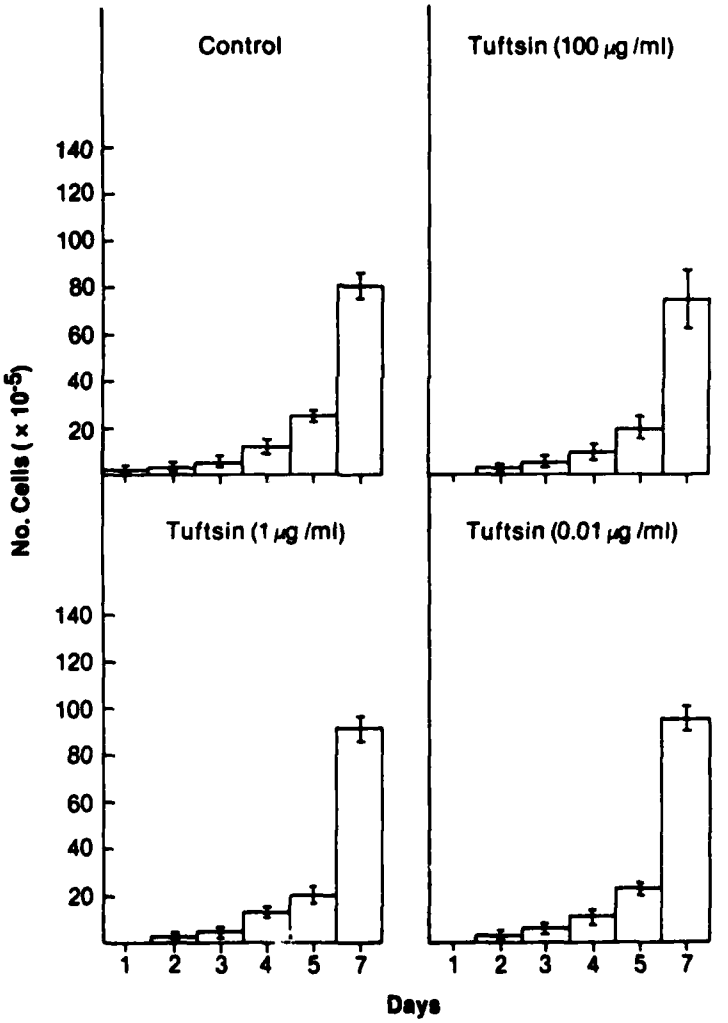


FIGURE 4. *Growth of tuftsin-treated K-Balb cells.* K-Balb 19a/h cells were plated at 5×10^4 cells per 6-cm dish in complete EMEM and were grown at 37°C for 24 hr. The cells were then refed on growth medium containing tuftsin. The cultures were detached by trypsinization and counted by trypan blue exclusion. The cell numbers represent the mean and standard deviation as determined from three dishes in duplicate experiments.

Since the p12 Rauscher leukemia virion protein has the same sequence at the amino-terminal end as tuftsin,[42] it may have a regulatory function in virus expression for the p12 protein. The present system provides a means to compare the mechanism of tuftsin action on phagocytic and fibroblastic cells, as well as a way of examining how small-molecular-weight peptides control cell growth and modify gene expression.

SUMMARY

The exposure of a high-passage clone of Kirsten sarcoma virus transformed Balb/c (K-Balb) mouse cells to tuftsin (Thr-Lys-Pro-Arg) enhanced the expression of endogenous xenotropic retrovirus. The tetrapeptide increased the expression of virus that was infectious for rat, but not mouse, cells in a concentration-dependent fashion (0.001–1000 μg/ml). Increased virus expression could be achieved during short-term incubations (3–4 hr), with maximum enhancement occurring over longer time periods (16–18 hr). The enhancement of virus expression by tuftsin was proportional to the spontaneous release of virus. The infectivity of the enhanced virus was neutralized by goat anti-RLV gp70 serum. Actinomycin D inhibited the induction of virus, suggesting that enhanced expression required *de novo* RNA synthesis. Tuftsin stimulated DNA, RNA, and protein synthesis in K-Balb cells during 16-hr incubations. Increased cellular proliferation was also seen at various time periods. The effects observed using K-Balb cells offer an opportunity to study the modulation of gene expression by tuftsin in a fibroblast culture system.

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Purine Salvage Pathway Enzyme Activity in Tuftsin-stimulated Macrophages

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The regulation of metabolic flux to produce superoxide ions (O_2^-) in phagocytes in response to chemotactic and phagocytic stimuli has not been fully elucidated. Oxidation of NADPH has been discussed as a source of O_2^-,[1] but we have been the only ones so far to evaluate the significance of O_2^- production during purine metabolism by xanthine oxidase (FIG. 1). Several investigators have made observations that relate purine metabolism to immunological activity. In addition to the considerable literature that relates adenosine deaminase (ADA) and purine nucleoside phosphorylase activities to the magnitude of an immune response, phagocytosis has been shown to be accompanied by increased uric acid excretion by macrophages,[2] infection has been observed to be associated with increased xanthine oxidase activity,[3] elicited macrophages have been found to contain about twice as much adenosine deaminase activity (per mg protein) as resident cells,[4] and ADA has been associated with maturation of monocytes into macrophages[5] and with normal chemotaxis.[6] We have shown previously that intracellular ADA activity in elicited macrophages increases in direct proportion to O_2^- secretion during phagocytosis and membrane perturbation by several soluble agents.[7-9] In addition, we have been able to demonstrate the localization of ADA activity in the macrophage membrane near phagocytic vacuoles by electron microscopy.[10]

We have used tuftsin as one of the acute stimulants for O_2^- production, and compared our findings to those of phagocytosis experiments of Najjar and others.[11] Unless stated otherwise, the cells used in our studies were peritoneal exudate cells (PEC) elicited with thioglycollate in ICR Swiss mice of the Roswell Park colony, and salvaged five days after thioglycollate injection as described previously.[7,8] On the average, 5×10^7 cells are obtained from a mouse. The cells are 80% large vacuolated mononuclear cells with typical macrophage morphology, and 20% lymphocytes. Erythrocytes, which contain high titers of superoxide dismutase activity, were eliminated by washing with buffered ammonium chloride. Because of the inverse relationship between O_2^- secretion and cell density,[9,12] we conducted our experiments at a density of 10^5 cells suspended per ml, the lowest density at which our experimental approach allows reliable O_2^- detection. In all experiments, the amount of extracellular O_2^- released was calculated from the amount of superoxide dismutase-free cytochrome *c*[13] reduced by use of the equation $\Delta E_{550nm} = 2.1 \times 10^4 M^{-1} cm^{-1}$.

The dose-response relation of tuftsin has been reviewed by Najjar[11] with the response expressed as the number of cells that contained phagocytized particles. Cells from several species, attached to cover slips or in suspension at densities up to 13×10^6 per ml, and phagocytizing a variety of particles, all showed half-maximum stimulation at about 50 ng per ml, i.e., 100 nM tuftsin, with the maximum response maintained to about 10 μM tuftsin. Accordingly, we have explored O_2^- secretion between 0 and 600 nM tuftsin.[14] The results are shown in FIGURE 2 in terms of O_2^- production with time, and in FIGURE 3 in terms of O_2^- produced after incubation for one minute in relation to tuftsin concentration. Because of endogenous superoxide dismutase activity, extracel-

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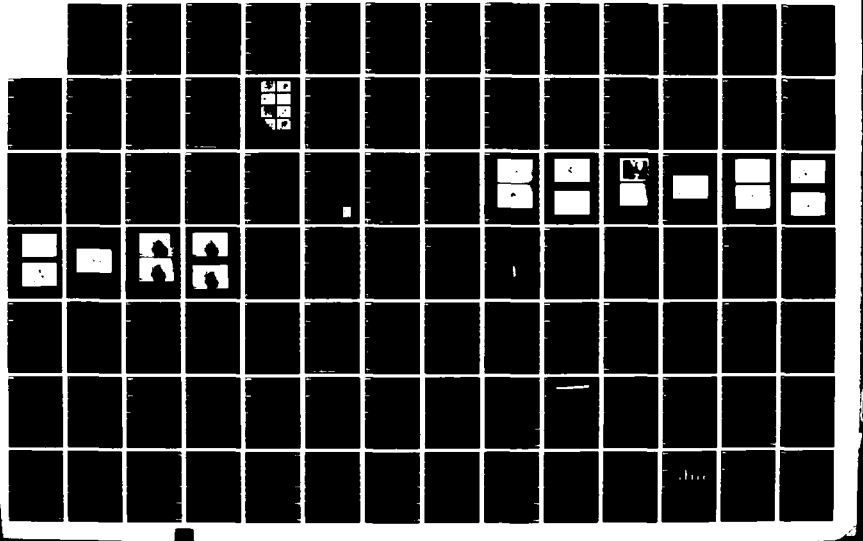

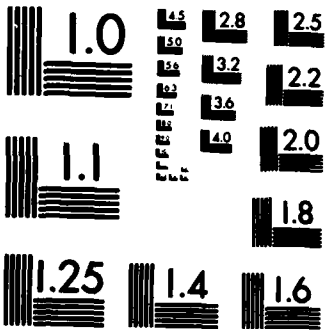

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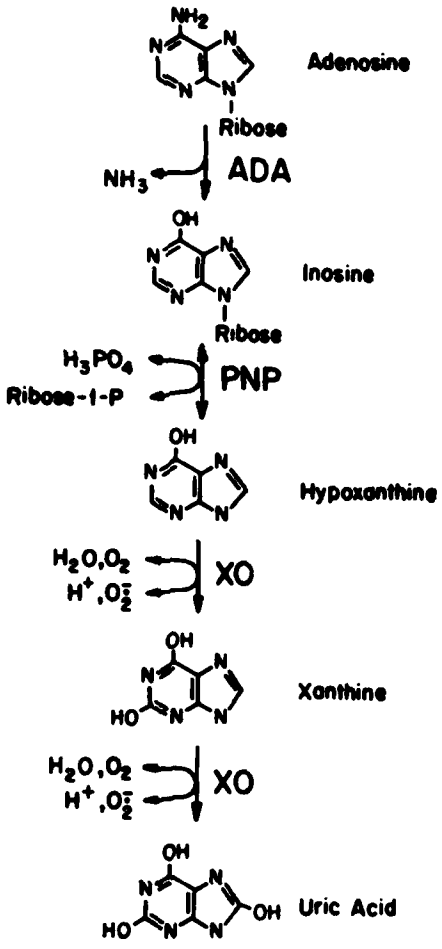

FIGURE 1. Purine catabolism.

lular O_2^- would be expected to be less than the O_2^- produced by the cells, and our values should be considered as minima. Superoxide secretion increased in direct proportion to tuftsin concentration to 400 n*M*. At 400 n*M*, near maximum O_2^- secretion of 18 nmole per minute per 10^6 cells was observed, a number in good agreement with that observed in Karnovsky's laboratory for phagocytosis (133 ± 35 nmole per minute per 10^7 cells).[1] Above 400 n*M*, there is a striking decrease in O_2^- secretion to almost base line levels. Phagocytosis does not decrease at elevated tuftsin concentrations but remains at

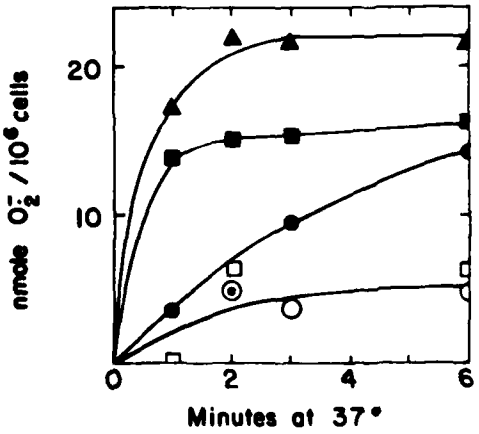

FIGURE 2. Time course of O_2^- production in the presence of the following tuftsin concentrations (n*M*): 125 (●), 250 (■), 375 (▲), 500 (○), 625 (□). Taken from Tritsch & Niswander.[14]

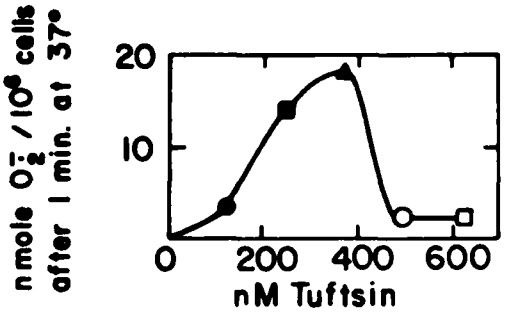

FIGURE 3. Dose-response curve after 1 minute of incubation at 37°. Data are taken from FIGURE 2. Taken from Tritsch & Niswander.[14]

maximum to 10 u*M*.[11] This suggests that at elevated tuftsin concentrations *in vivo*, phagocytized particles may not be killed by the oxygen-free radicals, with the consequence that the phagocytes would function to spread viable pathogens within the organism.

After completion of the O_2^- secretion experiments, we examined intracellular ADA activity. The cells were lysed by freezing and thawing in 0.5% Triton X-100 as previously described.[7,8] The detergent serves to solubilize membrane-associated enzyme. For ADA assay, 0.1 ml of 1 m*M* adenosine, 0.15 ml of 1.062 *M* Tris buffer, pH 7.3, and 0.25 ml water were mixed and placed into a 1-cm light path cuvette thermostated at 37° in a Cary model 14 spectrophotometer, 0.5 ml of cell lysate added, which is equivalent to 10^5 cells, and the rate of decrease in absorbance at 265 nm was recorded. A change in 1.0 absorbance is equivalent to 0.13 μmole adenosine deaminated.[15]

As shown in FIGURE 4, intracellular ADA activity was directly proportional to secreted O_2^-. It was of no apparent consequence whether tuftsin levels were used that produced near maximum O_2^- secretion, or whether the effect was caused by so high a concentration of tuftsin that near base line O_2^- secretion was observed. Furthermore, a linear regression calculated by the method of least squares through these data was

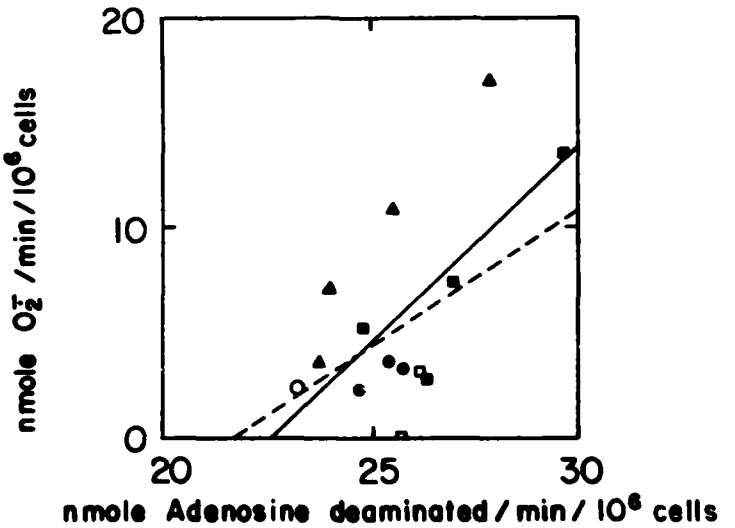

FIGURE 4. Relation between O_2^- secretion and intracellular ADA activity. Data taken from FIGURE 2. Solid line was calculated by the method of least squares. Broken line is taken from Tritsch & Niswander[7] relating O_2^- and ADA during stimulation of PEC by 250 n*M* tuftsin, the chemotactic peptide formyl-L-Methionyl-L-Lysyl-L-Phenylalanine, cytochalasin E + Phaesolus vulgaris lectin, and phagocytosis of opsonized zymosan. Taken from Tritsch & Niswander.[14]

similar to data obtained previously[7,8] with different chemotactic and phagocytic stimuli. This observation led to our suggestion that the relation between O_2^- and ADA is independent of the nature or magnitude of the stimulus used to elicit the effect.

The data in FIGURE 4 do not allow calculation of stoichiometry between O_2^- production and metabolic flux through the purine catabolic pathway because of endogenous superoxide dismutase activity. However, it is evident from the figure that the observed increase in ADA activity would be sufficient by itself to account for the concomitant increase in O_2^- production.

Because of the previously alluded to production of O_2^- through NADPH oxidation, we have examined the relative importance of these two pathways in O_2^- secretion by tuftsin-stimulated macrophages.[16] Peritoneal exudate cells (10^5 per ml) were incubated at 37° for 15 minutes before tuftsin addition in 0.1 m*M* adenosine to stimulate metabolic flux through the purine pathway, in 0.03 m*M* allopurinol (hydroxypyrazolo(3,4-D)pyrimidine) to inhibit xanthine oxidase, and a mixture of the two. After stimulation with 375 n*M* tuftsin, O_2^- secretion as well as intracellular ADA and NADPH oxidase activities were determined and compared to data from tuftsin-stimulated cells incubated in glucose containing balanced salt solution only. As seen in TABLE 1, relative to the control experiment (#1), adenosine more than doubled O_2^- release, allopurinol approximately halved it, and the particular combination of adenosine and allopurinol we used essentially neutralized the effects of each when used individually. ADA changes were small. No NADPH oxidase activity could be detected; more than 10^5 cells are generally used to detect this enzyme activity. When lysates of 10^7 cells were used, NADPH oxidase activity could readily be demonstrated. Thus, O_2^- production attributable to NADPH oxidase could be no more than 10% of that attributable to ADA.

It has been reported[17] that the Michaelis constant (K_m) of NADPH oxidase decreases from 4.0 for resting cells to 0.4 m*M* NADPH when the cells are allowed to phagocytize particles. We determined K_m of ADA from resting and tuftsin-treated PEC elicited with thioglycollate as well as with casein. No statistically significant difference was found in K_m for adenosine. In contrast to NADPH oxidase, ADA exists as a mixture of several isoenzymes that are in equilibrium with each other. Thus, possible alteration in K_m for only one of the isoenzymes might not be experimentally detectable. However, we were able to show that the heat stability of ADA from tuftsin-stimulated cells was increased in thioglycollate as well as in casein-elicited cells

TABLE 1. Effects of Adenosine and Allopurinol on Superoxide Production

Exp. No.	Addition During[a] Preincubation	Extracellular[b] O_2^-	Intracellular	
			ADA[c]	NADPH Oxidase[d]
1	None	1.59	4.3	<0.32
2	Adenosine	3.70	3.8	<0.32
3	Allopurinol	0.70	5.7	<0.32
4	Adenosine + Allopurinol	1.40	4.2	<0.32

[a]Each mixture was 3.0 ml in total volume, contained 101,000 PEC/ml, and was 120 μM in cytochrome *c*. After 15 min preincubation at 37°, the reactions were started by the addition of tuftsin as described in the text.

[b]nmole O_2^-/min/10^6 cells.

[c]nmole adenosine turned over per min per 10^6 cells.

[d]nmole NADPH turned over per min per 10^6 cells.

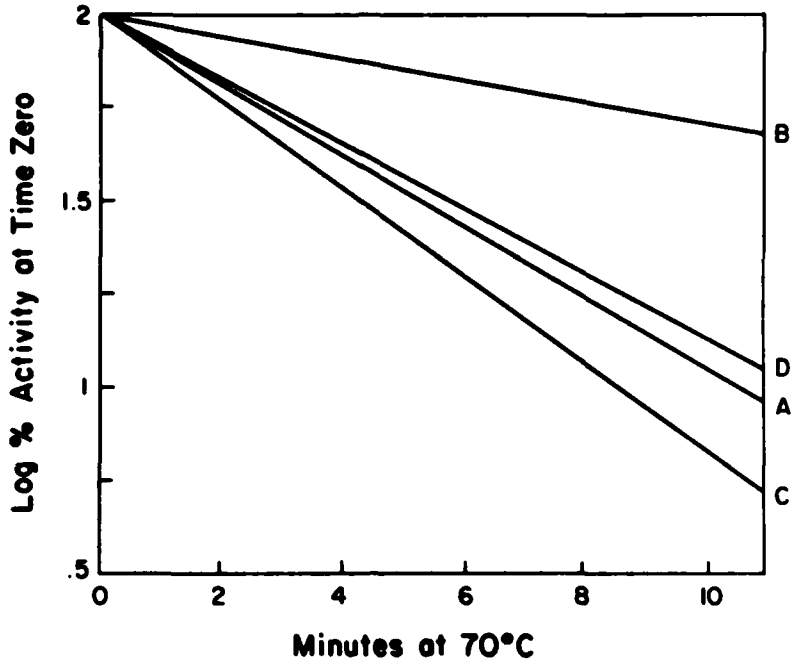

FIGURE 5. Rate of heat inactivation at 70° of ADA activity from PEC elicited with thioglycollate and stimulated with tuftsin (A), unstimulated (B), elicited with casein and stimulated with tuftsin (C), unstimulated (D). For clarity, the data points are not shown. The lines were computed with a linear regression model that assumes sigma proportional to x and the y intercept equal to 2. Computational details may be found in Snedecor.[18]

(FIG. 5). Thus, tuftsin stimulation influences ADA polymorphism in some as yet undetermined manner.

CONCLUSION

Our findings are applicable in a strict sense only to the experimental system employed in these studies. It is hoped however, that thioglycollate-elicited mouse PEC are sufficiently typical of this cell type that some general conclusions about the nature of the control of metabolism during chemotactic and phagocytic stimulation of phagocytes may be justified.

Tuftsin stimulation of elicited cells results in activation of purine catabolism, which directs metabolic flux through xanthine oxidase to produce superoxide anions. In our experimental system, this appears to be the source of at least 90% of the superoxide. Hence, oxidases for reduced pyridine nucleotides are not the key enzymes in this context in these cells. The ability of the xanthine oxidase reactions to produce this O_2^- appears to be related to ADA activity regulation of metabolic flux towards hypoxanthine formation. The sensitivity of the enzymes of this pathway to inhibition by a number of agents, especially many of those used in chemotherapy of neoplastic disease, suggests that impaired macrophage function may be part of the basis for the immunosuppressive side effects of several of these therapies. Furthermore, because macrophages exercise broad influence on the regulation of normal immune function, we propose that our findings account, at least in part, for the need for adequate ADA

activity for normal immune function, and may form the basis of the association of ADA deficiency with immunodeficiency.

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Tuftsin Receptors[a]

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INTRODUCTION

Structure-function studies with a multitude of tuftsin analogues have clearly demonstrated that even minute molecular alterations of the parent peptide may very often lead to the diminishment or abolishment of biological activity, or may even turn the peptide into a tuftsin inhibitor (for review see refs. 1, 2). This was observed, for example, in most NH_2-terminal modifications, that is, involving the Thr^1-residue, with many within-chain substitutions of various amino acids, or in analogues in which the positive charge of the Arg^4-residue was partially hindered or slightly removed, either closer or further from the main peptidic backbone.[1,3] These findings, illustrated with polymorphonuclear leukocytes (PMN-leukocytes), monocytes, and macrophages, strongly pointed at the existence of a specific receptor site for tuftsin on phagocytic cell surfaces.

The presence of cellular receptors for tuftsin was also suggested by an earlier work of Constantopoulos and Najjar.[4] These investigators have shown that treatment of PMN-leukocytes with the enzyme neuraminidase led to the abolishment of the phagocytosis-stimulating activity of tuftsin, while cell viability is still preserved. This finding, however, may be interpreted merely on the basis of prevention of some nonspecific electrostatic association between the negative cell surface and the rather highly positive peptide.

[a]Abbreviations for amino-acid derivatives and peptides follow the IUPAC-IUB Commission of Biochemical Nomenclature Symbols, see Eur. J. Biochem. 1979. 27: 201–207.

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Obviously, direct binding studies, using labeled tuftsin analogues, for example, those carrying radioactive or fluorescent probes, should be employed in order to prove the existence of specific cellular loci for the peptide. These derivatives should be as close as possible to tuftsin and possess high tuftsin-like activity in a variety of biological assay systems.

SYNTHESIS OF TRITIATED TUFTSIN

On the basis of the above considerations, we have synthesized tritium-labeled [^{3}H]tuftsin, using three different routes. The first, shown in FIGURE 1, is based on the coupling of 5-^{3}H-arginine with large molar excesses (~100 fold) of Boc-Thr-Lys(Z)-Pro-ONSu. Incorporation of the tritiated arginine into the peptide chain is high. The crude [^{3}H]tuftsin obtained after an appropriate deprotection is purified by ion-exchange chromatography on a Dowex 50W-X4 column, yielding electrophoretically and HPLC (C-18 reversed phase column) pure [^{3}H]tuftsin possessing full tuftsin-like activity in overall yield of 42%.[5] Preparation with specific activities of 9–24 Ci/mmol were obtained in this manner, depending on the specific activity of the ^{3}H-arginine employed.

The second route to [^{3}H]tuftsin (FIG. 2) is rather similar to the previous one and involves the coupling of 2,3-^{3}H-arginine with the tripeptide active ester Boc-Thr-Lys(Boc)-Pro-ONSu (P. Gottlieb and M. Fridkin, unpublished data). An electrophoretically and HPLC-pure product of specific activity of 9–24 Ci/mmol is usually obtained by this route in about 68% overall yield. The product is identical to tuftsin in all physicochemical, as well as biological, characteristics. The advantage of the synthetic route over the one described in FIGURE 1 lies in the final convenient, single-step, deprotection with trifluoroacetic acid.

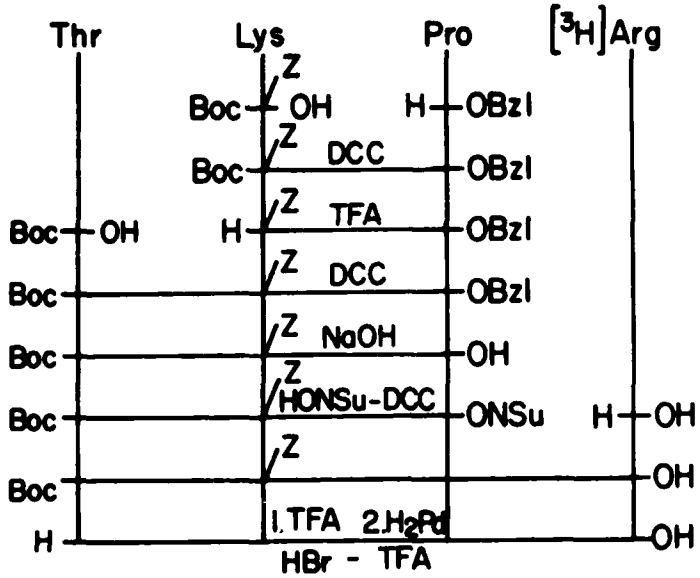

FIGURE 1. Synthesis of [^{3}H-Arg^4]tuftsin. ^{3}H-Arg= 5-^{3}H-arginine.

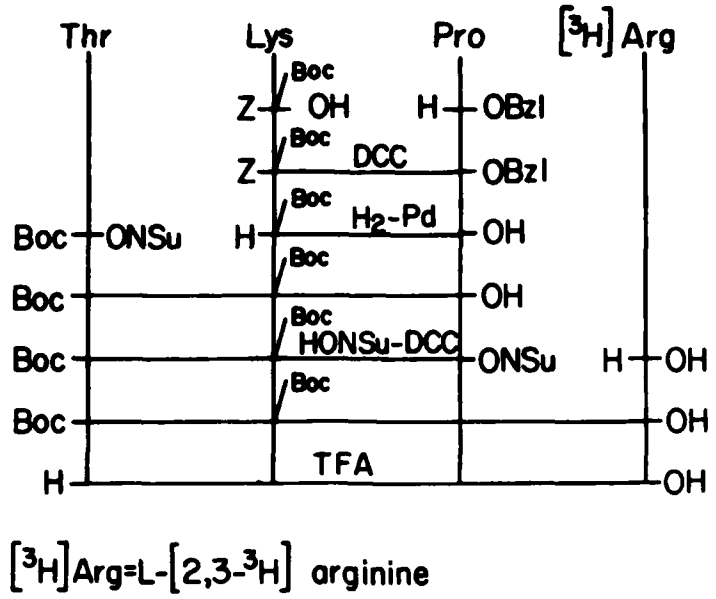

FIGURE 2. Synthesis of [^{3}H-Arg^4]tuftsin. ^{3}H-Arg= 2,3-^{3}H-arginine.

The third synthetic route to tritiated tuftsin is depicted in FIGURE 3. This pathway is based on the preparation of a [3,4-dehydro-Pro^3]tuftsin ([ΔPro^3]tuftsin) that can be easily purified and consequently catalytically (PdO) reduced by tritium gas to yield [^{3}H-Pro^3]tuftsin identical in its physicochemical and biological features to tuftsin.[6] Specific activities of products depend on the water or protic content, that is, extent of possible T $\rightarrow$ H exchange, of the reduction mixtures and on the isotopic purity of the T_2-gas employed. Values of specific activity of 5–15 Ci/mmol can be obtained by this procedure.

BINDING OF [^{3}H]TUFTSIN TO PHAGOCYTIC CELLS

A typical binding assay[7] consists of preparation, usually in Falcon tissue culture dishes, of cell monolayers that are washed several times with phosphate-buffered saline (PBS) and incubated, at 22°C for 35–45 minutes, with increasing concentrations of [^{3}H]tuftsin (having a specific activity of 9–24 Ci/mmol). The nonspecific component of the binding is assessed by a parallel incubation of cells with [^{3}H]tuftsin in the presence of 10^{-5} *M* unlabeled tuftsin. Subsequent to this incubation, the tuftsin-PBS solution is aspirated and the plates washed twice with PBS. Cell monolayers are dissolved in aqueous sodium dodecyl sulfate (0.1%) and the solutions obtained added to vials containing Triton-toluene mixture and radioactivity is then measured in a liquid scintillation spectrophotometer. Each point of the resulting binding-plots derives from triplicate cultures. Binding can also be performed on cell suspensions in test tubes.[5] Great care should be taken, however, of bindings of [^{3}H]tuftsin, as well as of unlabeled tuftsin to certain tube types. Such binding may sometimes reach high values.[6]

Using [^{3}H-Arg^4]tuftsin, we performed in our laboratory binding studies at 22°C on various blood cell types. Thus, it was manifestly observed that specific receptor sites exist for tuftsin on PMN-leukocyte cells.[5] Binding of the peptide to cells is a time-dependent (FIG. 4), reversible process. Full occupancy of available binding sites, as depicted in FIGURE 5, occur at a [^{3}H]tuftsin concentration of about 280 n*M* and

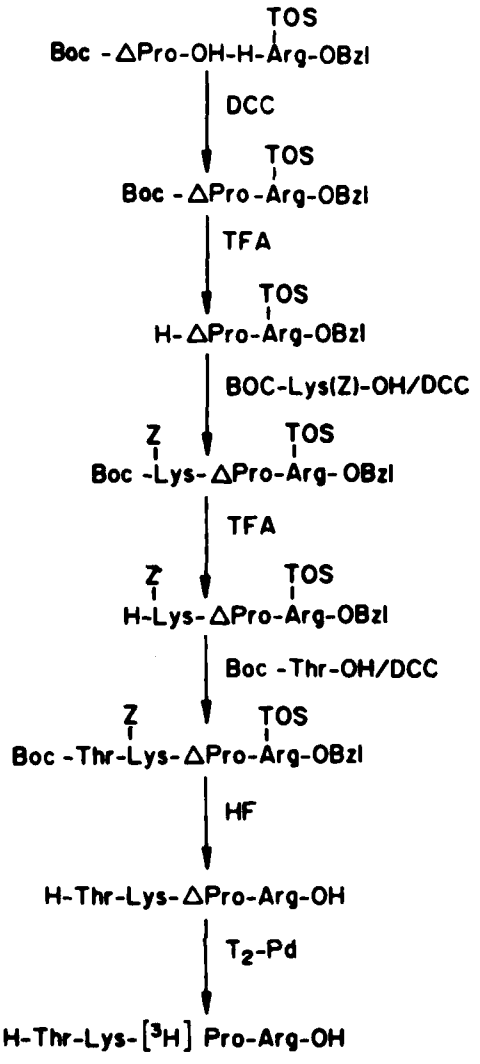

FIGURE 3. Synthesis of [^{3}H-Pro^3]tuftsin. ΔPro= L,3,4-dehydroproline.

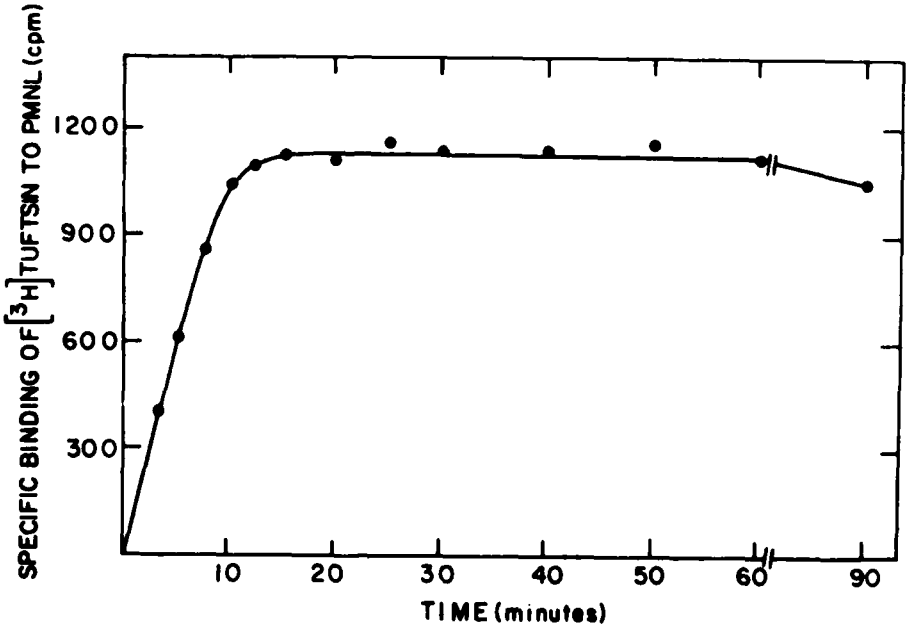

FIGURE 4. Time course of [^{3}H-Arg^4]tuftsin-specific binding to human polymorphonuclear leukocytes.[4] Assay performed at 22°C and at peptide concentration of 130 n*M*.

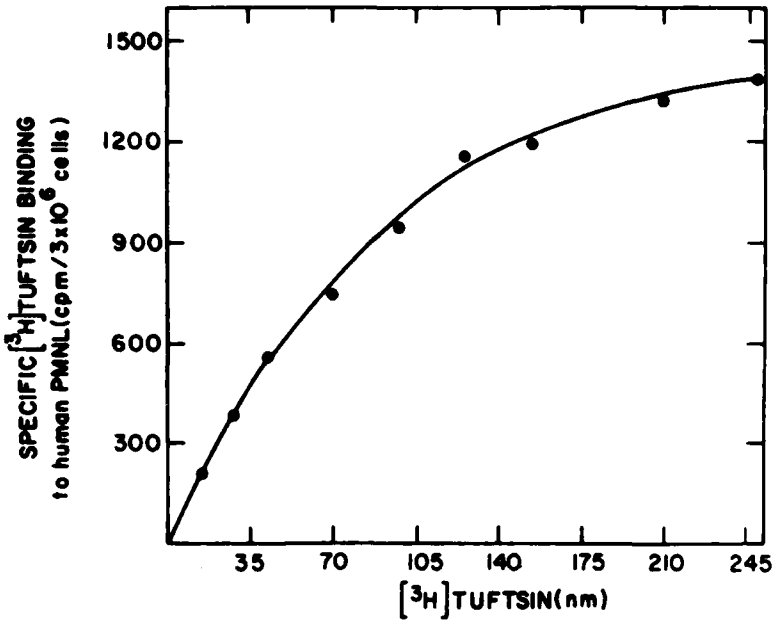

FIGURE 5. Specific binding of [^{3}H-Arg^4]tuftsin to human polymorphonuclear leukocytes as a function of concentration of the tritiated peptide.[5,9] Binding was performed for 45 minutes at 22°C.

Scatchard analysis of this data (FIG. 6) indicates that only one type of binding site exists for tuftsin. The calculated equilibrium dissociation constant, Kd, is 1.3×10^{-7} *M*, a value that is notably close to the levels of tuftsin that evoke a half-maximal biological response in human PMN-leukocytes.[1,8] Assuming an equimolar peptide-receptor-type complex, about 50,000 binding sites are present for tuftsin on each polymorphonuclear leukocyte cell.

FIGURE 7 displays the results of competitive binding of unlabeled tuftsin and of several synthetic analogues of tuftsin with [^{3}H-Arg^4]tuftsin to PMN-leukocytes, which strongly substantiate the receptor's specificity. The extent of binding-inhibition is in

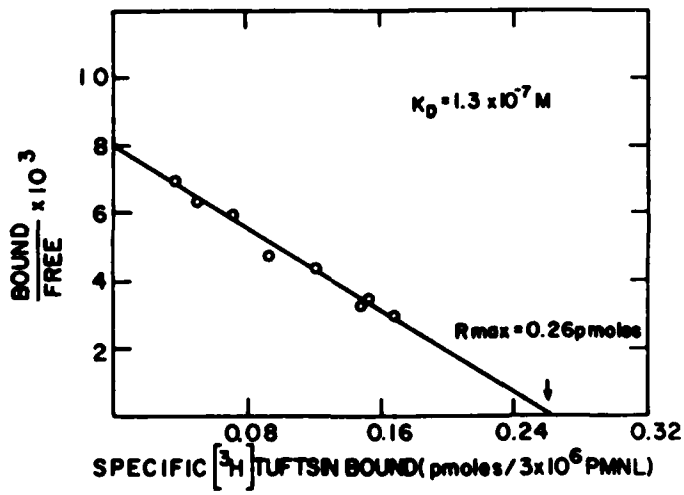

FIGURE 6. Scatchard-plot of specific [^{3}H-Arg^4]tuftsin-binding depicted in FIGURE 5.[5,9]

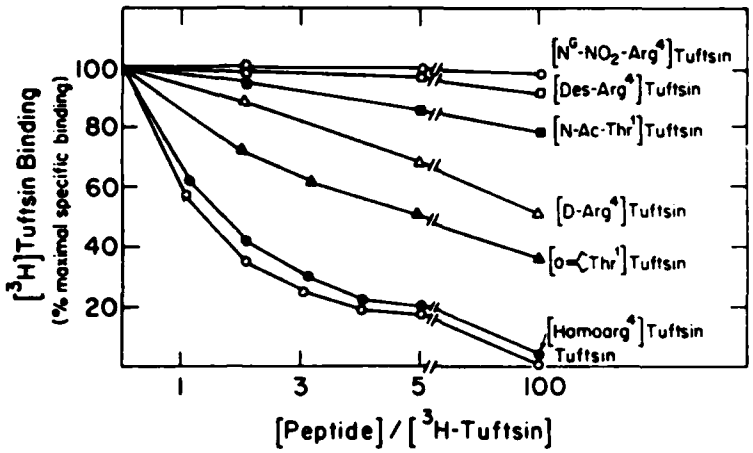

FIGURE 7. *Effect of unlabeled tuftsin and some of its synthetic analogues on the binding of* [^{3}H-Arg^4]tuftsin to human polymorphonuclear leukocytes.[5,9]

line with the tuftsin-like biological activity: agonistic of the peptide derivative or with its capacity, antagonistic, to inhibit tuftsin's functions.[1,5,9] It is worth pointing out that when the concentrations of unlabeled tuftsin and that of [^{3}H]tuftsin are equimolar, inhibition of binding is about 50% (FIG. 7).

Binding studies of [^{3}H-Arg^4]tuftsin to human monocytes (binding curves are not shown) demonstrated a specific binding with a Kd of 1.25×10^{-7} *M* and the existence of about 100,000 tuftsin sites per cell.[1,5,9] Purified lymphocyte preparations were found to bind less than 5% of tritiated tuftsin as compared with PMN-leukocytes.[1,5,9] This minute binding capacity may be interpreted as being due to low affinity of cell-tuftsin interaction or to the complete absence of specific sites for tuftsin. The existence, however, of a subpopulation of lymphocytes that possess tuftsin's receptor sites and may thus account for the threshold binding observed should not entirely be excluded. This last assumption may be viewed along with the findings of Nair *et al.*[10] These investigators, using tuftsin-^{14}C-methyl ester and ^{125}I-tuftsinyl-tyrosine, were able to show that specific sites for tuftsin exist on PMN-leukocytes and monocytes. The binding of the latter radiolabeled peptide, but not the former, to pure lymphocyte preparation was substantial and statistically significant.

The binding of [^{3}H-Arg^4]tuftsin to mouse peritoneal macrophages stimulated *in vivo* by a variety of sensitizer agents has also been studied in our laboratory.[7] Binding was found to be specific, rapid, and saturable (curves are not shown). The equilibrium constant, K_D, was approximately 5.3×10^{-8} *M*, as calculated from the Scatchard plot (not shown). Only one type of binding site exists, and the number of receptors calculated per macrophage cell is about 75,000. No meaningful difference in binding parameters could be detected between stimulated or nonactivated macrophages, or between cells sensitized *in vivo*, by different agents.[7] Competitive binding studies with unlabeled tuftsin, as well as a variety of synthetic tuftsin analogues demonstrate that the extent of peptide binding corresponds to its capacity to augment the phagocytic response of the macrophage cell.[7,9]

The specific binding of tuftsin to the macrophage-like line P388D1 was also reported.[11] The binding of tuftsin to various macrophage-derived cells and its biological implication will be discussed in detail in these proceedings by Gottlieb *et al.*

Human erythrocytes[5,9] and tertiary mouse embryo fibroblasts[7] were shown to be devoid of capacity to bind tritiated tuftsin.

ON THE NATURE OF THE TUFTSIN RECEPTOR

Tuftsin is part of the C_H2 domain of the the Fc-fragment of the γ-globulin protein. The possibility that the tuftsin binding sites on phagocytic cells are related in some way to the Fc-receptor was raised by us[1,5] and by Nair *et al.*[10] As an approach to examining this possibility, three peptides encompassing the tuftsin moiety and corresponding to the tuftsin region of the Fc-fragment were recently synthesized in our laboratory. The peptides prepared are:

H-Thr-Lys-Pro-Arg-Glu-Gln-Gln-Tyr-OH (positions 289-296; octa-C).
H-His-Asn-Ala-Lys-Thr-Lys-Pro-Arg-OH (positions 285-292; octa-N)
H-His-Asn-Ala-Lys-Thr-Lys-Pro-Arg-Glu-Gln-Gln-Tyr-OH (positions 285-296; dodeca)

The ability of the three peptides to compete with [^{3}H-Arg^4]tuftsin in binding to mouse peritoneal macrophages, at 22°C, was studied. As shown in FIGURE 8, all peptides can displace tritiated tuftsin from its cellular receptors similar to unlabeled tuftsin. This finding coupled with the observation that many analogues of tuftsin, some of which, particularly NH_2-terminal analogues, are only slightly different from tuftsin, cannot inhibit the binding of tritiated tuftsin to macrophages and other phagocytic cells,[1,9] point at the possibility that the tuftsin receptor can specifically accommodate "native" segments of the Fc-fragment. Moreover, as shown in TABLE 1, the three synthetic peptides are not only capable of binding to the tuftsin receptor, but are also similar to tuftsin in augmenting the phagocytic activity of macrophages. On the basis of the above-presented data, we are currently exploring two possibilities as to the nature of the tuftsin receptor. First, that it is a subclass of Fc-receptor (such classification has already been shown to exist[12]). Second, that a distinct receptor for tuftsin exists, which at one time was structurally related, but at present retains only some homologous features of the Fc-receptor.

The tuftsin molecule emerges from a unique protein fraction of IgG origin, leukokinin, probably through a several-step enzymatic releasing pathway. Although the mechanism of this release is not yet elucidated, the overall process leading to the final product, tuftsin, has been previously described (for review see refs. 1, 13, 14).

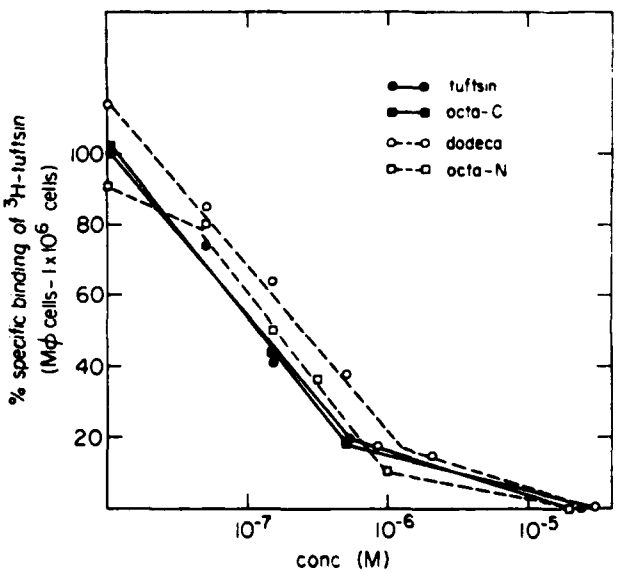

FIGURE 8. Effect of unlabeled tuftsin and its Fc-related extended analogues on displacement of [^{3}H-Arg^4]tuftsin from thioglycocollate-stimulated mice macrophages. Assay performed at 22°C at labeled-peptide concentration of 50 n*M*.

TABLE 1. Phagocytosis-stimulating Activity of Tuftsin and Extended Analogues on Ingestion of IgG-coated Sheep Red Blood Cells (SRBC) Labeled with ^{51}Cr by Macrophages[a]

Peptide (200 n*M*)	% Phagocytosis[b]
Tuftsin	61
Thr-Lys-Pro-Arg-Glu-Gln-Gln-Tyr	56
His-Asn-Ala-Lys-Thr-Lys-Pro-Arg	67
His-Asn-Ala-Lys-Thr-Lys-Pro-Arg-Glu-Gln-Gln-Tyr	55

[a]The assay was carried out essentially as described by Gottlieb *et al.*[20]
[b]Phagocytosis index calculated as follows:

$$\frac{\text{cpm of stimulated cells} - \text{cpm of unstimulated cells}}{\text{cpm of unstimulated cells}}$$

The possibility that an intermediate process is involved in the generation of tuftsin, namely formation of larger, pretuftsin, peptidic fragments, has not been described. Thus, to ascertain whether the ability of the two octapeptides and the dodecapeptide to bind to and to activate macrophages is their intrinsic feature, or due to enzymatic breakdown that eventually yields tuftsin, the peptides were incubated for different periods of time, at both 22°C and 37°C, with the cells. Careful analysis of the incubation mixtures by HPLC could not demonstrate the formation of tuftsin, nor the formation of either of its two NH_2 or COOH-terminal tetrapeptide extensions. Upon prolonged incubation, however, a very small extent of "nonspecific" peptide degradation was noticed. As shown in FIGURE 9, the method can clearly distinguish between the various relevant peptide derivatives. In view of these results, the existence *in vivo* of some Fc-derived tuftsin-containing sequences, which may play a certain physiological role, should not be excluded.

The formyl-chemotactic peptide, f-Met-Leu-Phe[15] (for review see refs. 16, 17), and tuftsin (for review see ref. 1) can stimulate several common cellular responses of

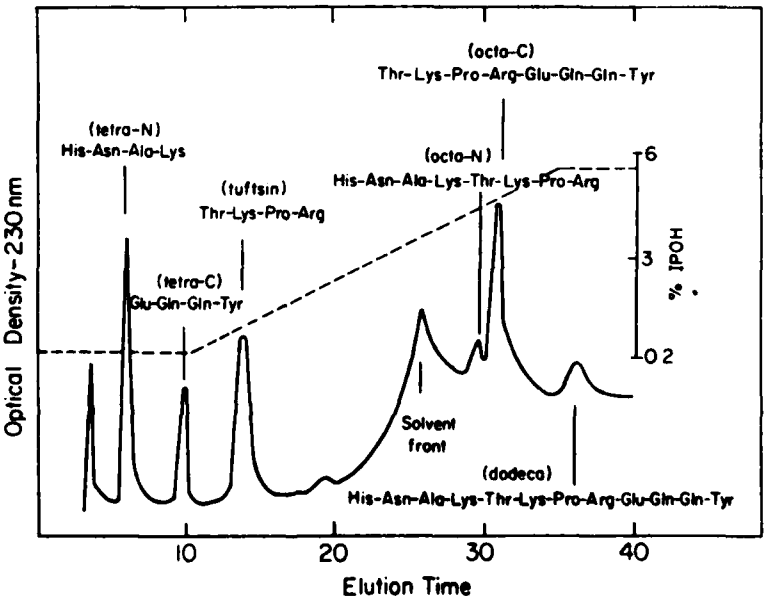

FIGURE 9. Separation of tuftsin and Fc-related peptides by high-pressure chromatography system (HPLC; reversed-phase C-18 column).

phagocytes. Thus for example, both peptides can augment oxidative respiratory burst and metabolism, increase locomotion, redistribution of cations, especially Ca^{++}, and lysosomal enzyme release of cells. It has been shown that both peptides initially bind to specific receptor sites, an association that is subsequently translated into the above cellular events (e.g., refs. 1, 17–19). It was of interest to examine whether the receptors of the two peptides share some common determinants. Consequently, the effect of the chemotactic peptide and its conjugate with tuftsin, H-Thr-Lys-Pro-Arg-Gly-Lys'-Gly-Phe-Leu-Met-NH-CHO (tuftsin-CP), on the inhibition of binding of [^{3}H-Arg^4]tuftsin to its receptor was studied. As shown in FIGURE 10, both peptides are capable of decreasing the initial amount of [^{3}H-Arg^4]tuftsin bound to human monocytes and of its displacement from cells. This latter effect was, however, much more pronounced with unlabeled tuftsin.

How can one explain the results of the competitive binding assay? As a major chemical difference exists between the formyl-chemotactic peptide and tuftsin (the first is a rather hydrophobic and neutral molecule, while the second is a very hydrophilic and positively charged species), it is difficult to assume that they share the same binding sites. One can argue, of course, that some topographical proximity exists

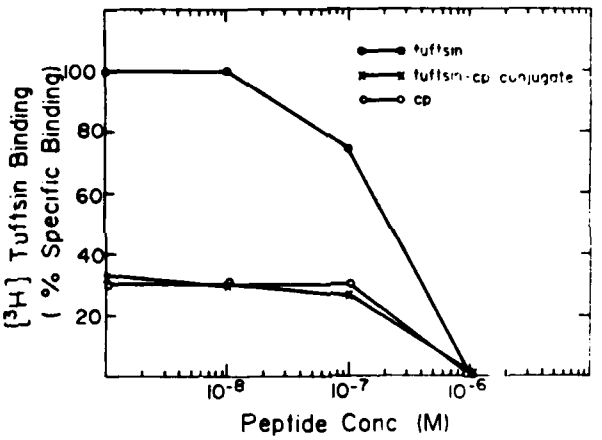

FIGURE 10. Effect of unlabeled tuftsin, its conjugate with formyl chemotactic peptide (tuftsin-CP; H-Thr-Lys-Pro-Arg-Gly-Lys'-Gly-Phe-Leu-Met-NH-CHO), and of N-formyl-Met-Leu-Phe-Gly (CP) on the binding of [^{3}H-Arg^4]tuftsin to human monocytes. Assay performed at 22°C at labeled-peptide concentration of 100 n*M*.

between the tuftsin and the f-Met-Leu-Phe receptors. A simpler explanation seems to us, however, more plausible. It is assumed that the interaction of both the chemotactic peptide[19] and tuftsin[1] with their receptors markedly affect cytoskeletal array, and cause a considerable overall change in cell surface. Such alterations, when brought about by one peptide, may lessen and weaken the binding of the other.

TUFTSIN RECEPTOR SITES ARE REGULATED

In order to explore certain features of the tuftsin receptor on the molecular and cellular levels, we have synthesized a number of its analogues that carry different probes at the COOH-terminus of the peptide.[20] We have previously shown that only the COOH-terminal extension of tuftsin's backbone leads to analogues that possess substantial tuftsin-like biological activities.[3] As we have seen in the earlier paragraphs of this article, NH_2-terminal extensions that are relevant to the sequence of the Fc-fragment may also fall into this category. Thus, we have synthesized: (1) photoaffinity-labeling analogues for the specific covalent attachment to the tuftsin

receptor, H-Thr-Lys-Pro-Arg-Tyr-Trp(NAPS)-OH (NAPS = 2-nitro-4-azido phenysulfenyl) and H-Thr-Lys-Pro-Arg-Trp(NAPS)-Tyr-OH;[20] (2) biotin analogue for separation and purification of the receptor by affinity methods, H-Thr-Lys-Pro-Arg-NH-$(CH_2)_2$-NH-Biotin;[20] and (3) fluorescent analogues containing either dansyl or rhodamine probes for microscopic visualization of the tuftsin receptor, H-Thr-Lys-Pro-Arg-NH-$(CH_2)_2$-NH-Dansyl,[20] H-Thr-Lys-Pro-Arg-Gly-NH-$(CH_2)_2$-NH-Rhodamine,[20] H-Thr-Lys-Pro-Arg-Gly-Lys(N^{ϵ}-Rhodamine)-OH[20] and H-Thr-Lys-Pro-Arg-Gly-Lys(N^{ϵ}-Tetramethylrhodamine)-OH (P. Gottlieb and M. Fridkin, unpublished data). The synthesis of the latter fluorescent peptide is depicted in FIGURE 11. The fluorescently labeled peptide possesses characteristic spectroscopic features as expected from a tetramethylrhodamine peptide-derivative, with excitation at 550 nm and maximal emission at 573 nm.[21] As shown in FIGURE 12, it is capable of inhibiting the binding of [^{3}H-Arg^4]tuftsin to its specific receptor sites on macrophage cells. The binding affinity of the fluorescent analogue to the receptor is somewhat lower (100 n*M*) than that of tuftsin itself (50 n*M*). The fact that the peptide derivative specifically recognized the tuftsin receptor, coupled with its ability to stimulate the macrophage cell to phagocytize opsonized sheep red blood cells, confirms the fact that it is a tuftsin-like active molecule. The other peptide derivatives reported above are as capable of binding to the macrophage and of augmenting its phagocytosis response.[20]

The tuftsin-rhodamine derivative, H-Thr-Lys-Pro-Arg-Gly-Lys(N^{ϵ}-Tetramethyl rhodamine)-OH, was used to envisage tuftsin receptors on surfaces of mouse peritoneal macrophage cells, employing the fluorescence image intensification technique. Association of cells with the fluoresence peptide occurred at 37°C, at a peptide concentra-

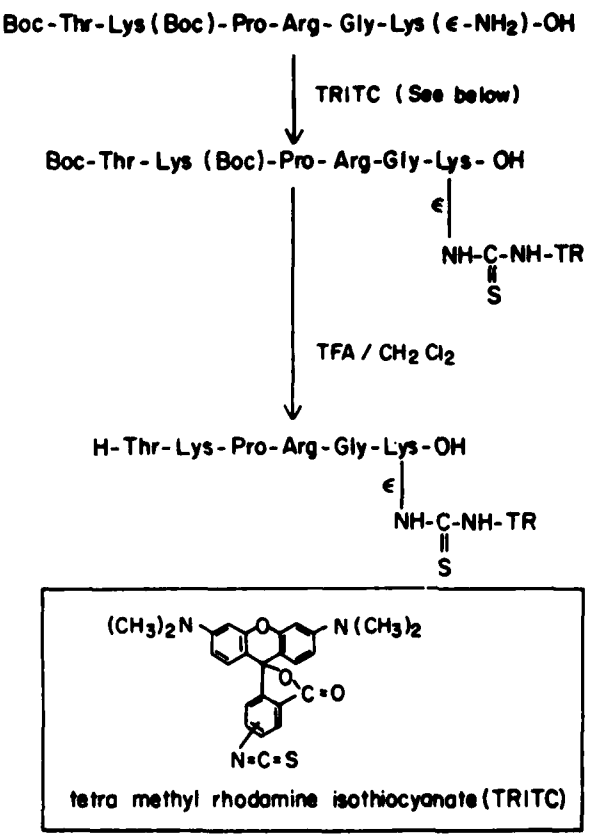

FIGURE 11. Synthesis of H-Thr-Lys-Pro-Arg-Gly-Lys(N^{ϵ}-Tetramethylrhodamine)-OH.

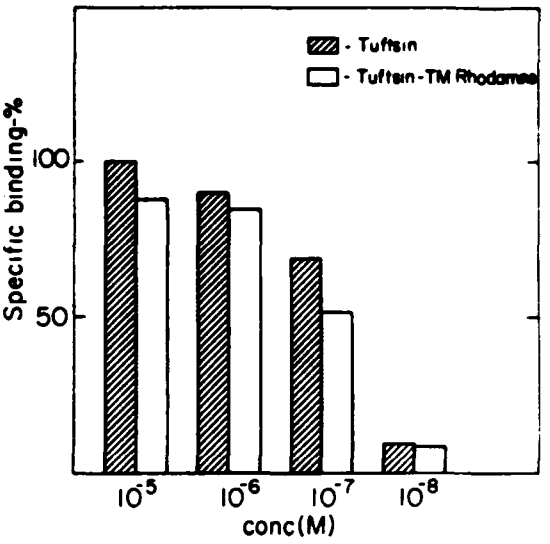

FIGURE 12. Competitive-binding inhibition of [^{3}H-Arg^4]tuftsin by unlabeled tuftsin and rhodamine-tuftsin analogue to mice macrophages.

tion of 2μmolar. Formation of peptide-receptor clusters and their subsequent internalization was found to be a rapid process (~5 minutes) (see FIG. 13). To further support the observation of the fast regulation of the tuftsin binding sites, quantitative evaluation of the peptide's receptor was carried out, using [^{3}H-Arg^4]tuftsin, after incubation of macrophages with an excess of unlabeled tuftsin for various time periods. This assay (FIG. 14) demonstrates that tuftsin sites are initially (~5–7 minutes) increased, and subsequently (~10–15 minutes) reduced in amount, thus confirming the fluorescent studies.

CONCLUSION

Tuftsin exerts stimulatory effects on various cellular events of phagocytic cells, the polymorphonuclear leukocyte, the monocyte, and the macrophage.[1,2,13,14] It seems reasonable to assume that association of the peptide with cells is initially electrostatic in its nature. It is then followed by a fast accommodation of tuftsin into specific receptor sites and a subsequent rapid internalization of the peptide-receptor complex. It is not yet clear whether tuftsin stimulates the cells before or after its penetration. As the cytoplasm of rabbit PMN-leukocytes was shown to contain proline peptidases capable of degrading tuftsin to its constituent amino acid,[22] the latter process might even be a means of peptide clearance.

Tuftsin was found to affect the intracellular levels of cyclic nucleotides, increase cGMP, and decrease cAMP in the PMN-leukocytes and macrophages.[1,23] This was found to be a rather rapid event (maximal response in about 10–20 minutes), and the effect of tuftsin on the intracellular concentration or distribution of Ca^{2+} was similar.[23] How and whether the two processes are connected to the triggering of the tuftsin receptor, that is, the mode of action of tuftsin, is still a matter for further investigation.

Aiming to shed further light on the nature of the tuftsin receptor, we are currently attempting its affinity labeling and isolation. This is done using the various synthetic derivatives of tuftsin described above.[20]

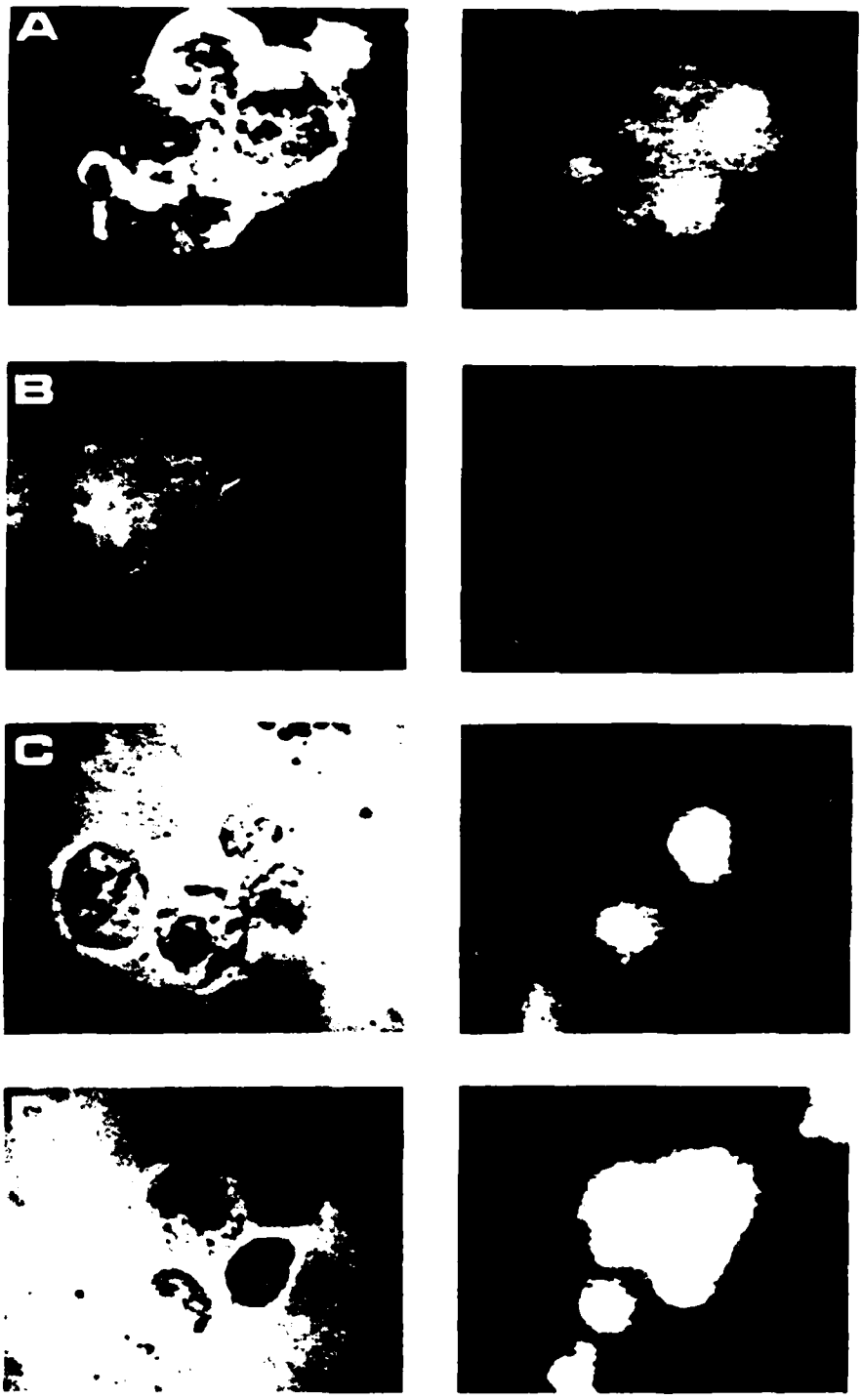

FIGURE 13. Fluorescent distribution of H-Thr-Lys-Pro-Arg-Gly-Lys(N'-Tetramethylrhodamine)-OH after incubation with macrophage cells. For each letter, a set of cells are shown in phase contrast (left) and fluorescence emission (right). Incubation was performed at 37°C.

(a) Uniform distribution of fluorescent tuftsin, at 200 n*M* concentration and incubation with cells for 5 minutes.

(b) Nonspecific binding of fluorescent peptide. Cells incubated as in (a), but in presence of an excess of tuftsin (20 μM).

(c) Cluster formation on cells after incubation with 200 n*M* of fluorescent analogue for 10 minutes.

(d) Internalized clusters after 10-minute incubation period using 2μM fluorescent-tuftsin. A similar pattern was observed after a 30 min incubation with 200 nm of fluorescent-tuftsin.

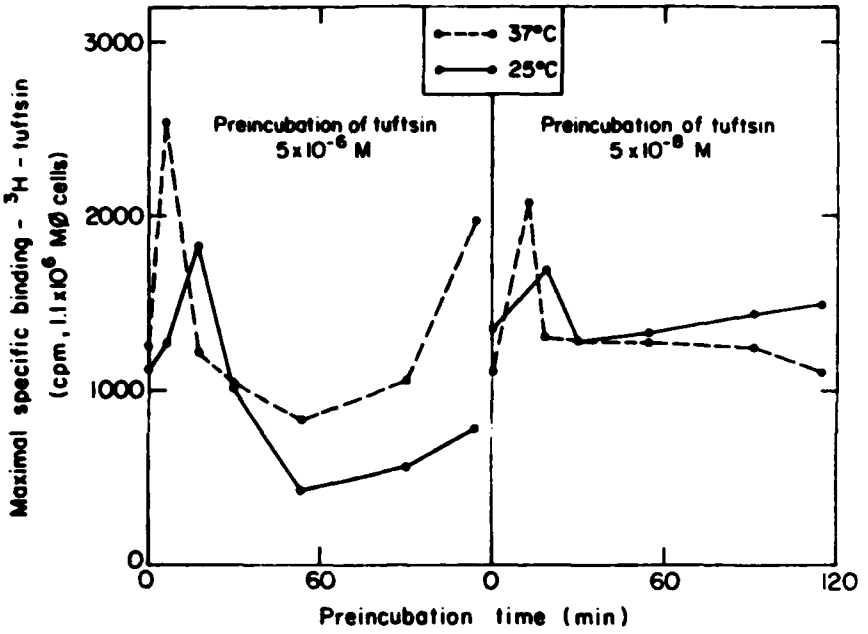

FIGURE 14. Effect of preincubation of tuftsin at a concentration of 50 nM and 5 μM on maximal binding of [^{3}H-Arg^4]tuftsin to macrophage cells.

ACKNOWLEDGMENTS

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Tuftsin Binding to Various Macrophage Hybridomas[a]

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INTRODUCTION

Macrophages participate in a wide variety of immunological processes, including phagocytosis, immune response through antigen presentation and lymphocyte activation, tumoricidal, and bactericidal activities. Despite their seemingly common cellular origin, macrophages do not consist of a homogeneous population. Macrophage subsets differ in morphology, expression of membrane markers, enzyme content, and biological activities.

The effect of tuftsin on the above biological activities has been and currently is the subject of investigation in a number of laboratories. These include stimulation of phagocytosis,[1,2] enhanced bactericidal[3] and antitumor activities both *in vivo* and *in vitro*;[4-6] and enhancement of antigen presentation capacity.[8]

Although much information has been gleaned concerning the activation of macrophages by tuftsin through specific cellular receptors,[9,10] little is known about the mode by which it is capable of stimulating the multitude of activities associated with tuftsin-macrophage interaction.

In order to further understand the specific controlling mechanisms of the many cellular immune responses in general, and the tuftsin effect in particular, separation and purification of macrophage subsets become an imperative step.

The vast variety of macrophages and the limitations of current separation methods has impeded the study of molecular mechanisms that regulate the above specific cellular activities. One approach to circumvent these problems is the development of macrophage cell lines by means of hybridization technology. The "immortalization" of various types of macrophages may offer experimental systems that are most suitable for analyzing controlling mechanisms of macrophages. We report the characterization of macrophage hybridomas. This will be correlated with the availability of tuftsin receptors. A number of implications about tuftsin activation will be discussed.

[a]This work was supported by Grant No. 2645 of the U.S.-Israel Binational Science Foundation, Jerusalem.

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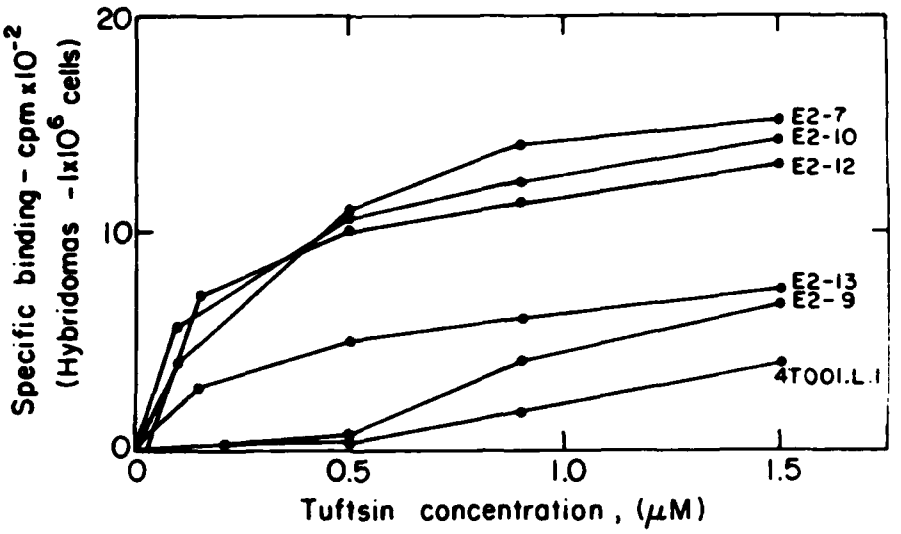

FIGURE 1. Competiton binding assay for tuftsin on hybrid cells. 50–100 n*M* ^{3}H-tuftsin ([^{3}H-Arg^4]tuftsin) was incubated with 1 × 10^6 hybrid cells. Indicated concentration of tuftsin was used.

GENERATION AND IDENTIFICATION OF MACROPHAGE HYBRIDOMAS

Macrophage and dendritic cells enriched from a splenic population[11] of C_3H mice were fused[12] with the myeloma cell line 4T00.1.L1.[13] Hybrids were selected by growth in HAT medium.[14] Rapid screening of the hybridomas was achieved by the presence of two macrophage-specific enzymes: lysozyme and nonspecific esterase.

Two out of the ten potential macrophage-like hybridomas were stained for the

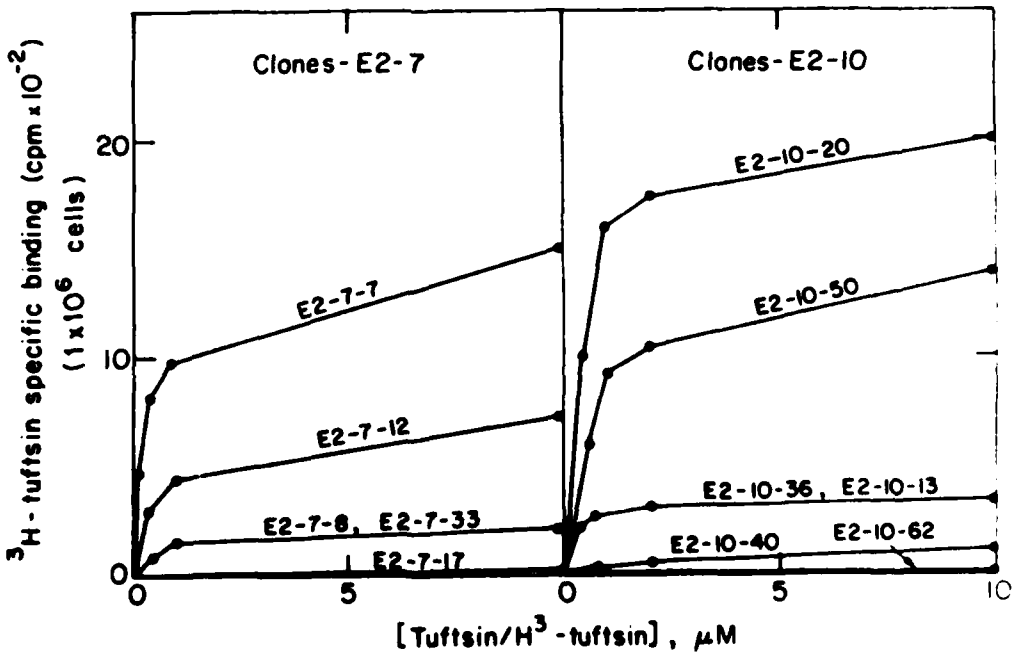

FIGURE 2. Competition binding assay for tuftsin on the clones produced from hybridoma (a) E2-7 and (b) E2-10. 50–100 n*M* ^{3}H tuftsin was incubated at 22°C for 30 min.

presence of the intracytoplasmic nonspecific esterase. These two hybridomas were designated E2-7 and E2-10. In parallel, cells were screened for the presence of tuftsin receptors using a competitive inhibition binding assay.[9,15] FIGURE 1 displays the results of this assay. It can be seen that hybrids E2-7, E2-10, and E2-12 displayed the greatest number of receptor sites with a similar dissociation constant previously reported for tuftsin. Other hybrid cells apparently bind, but with either many fewer receptor sites (i.e. E2-13), or with very small numbers of receptor sites and a lesser affinity (4T00.1L.1 and E2-9).

Morphologically, the E2-7 cell line exhibited the greatest resemblance to macrophages. The cell had an elongate ameboid, or a stellate appearance and adhered to culture dishes. Furthermore, E2-7 was found to secrete large amounts of lysozyme. The E2-10 hybrid, although esterase-positive and containing significant quantities of tuftsin receptor, appears to be morphologically similar to the myeloma cell, is not adherent, and did not secrete lysozyme in significant quantities.

Concentrating on these two hybrids, we cloned these cell lines in soft agar to remove contaminating hybrids and nonfunctional variants. The clones were then subjected to a competitive binding assay for tuftsin receptor. FIGURE 2(a,b) shows the binding patterns for tuftsin. Inspection of these graphs indicates that only E2-7-7, E2-7-12, and E2-10-20, E2-10-50 clones retain significant binding for tuftsin.

TABLE 1. Properties of Different Clones of MØ-Hybridomas

Clone	Fc Receptor[a] (%)	Ia-antigen[b] (%)	Tuftsin Binding[c]
E2-7-7	4	45	++
E2-7-12	3	51	+
E2-10-20	58	3	++
E2-10-62	65	0	–

[a]Fc receptor is assessed by rosette formation using opsonized erythrocytes.
[b]Ia-antigen was measured using indirect fluorescence serology.
[c]Tuftsin receptor was assayed using a binding competition assay.

MOLECULAR SURFACE MARKERS

Two cellular surface markers were used in differentiating the E2-7 and E2-10 clones from each other. These markers are the Fc receptor and the MHC-encoded Ia alloantigens. Fc receptor was detected by rosette formation with opsonized erythrocytes (EA).[16] Expression of Ia^k alloantigen was determined using indirect fluorescence serology.

Focusing on two clones (E2-7-7 and E2-10-20), it is clear that, upon comparison, these clones differ in their surface markers. TABLE 1 demonstrates that E2-10-20 in comparison to E2-7-7 has significant levels of Fc receptor. On the other hand, E2-7-7 stains positively for the Ia antigen, while E2-10-20 does not.

BIOLOGICAL ACTIVITY

Continuing with our effort to characterize these clones, E2-10-20 and E2-7-7 were subjected to a phagocytosis assay and their ability to present antigen to T-cells.[8]

The phagocytosis assay was performed using opsonized sheep red blood cells (EA). Erythrocytes were initially incubated with anti-sheep erythrocytes of the IgG class at

37°C for 45 min in a final dilution of 1:600. After extensive washings, cells were then incubated with ^{51}Cr for 2 hrs at 37°C and again washed extensively. Using a concentration of 0.5% labeled EA, phagocytosis was performed for 1 or 2.5 hrs at 37°C.

Cells were washed and treated with 0.83% ammonium chloride to remove attached and free-labeled EA. As expected (FIG. 3), the clone E2-10-20 that was rich in Fc receptor was highly phagocytic, while clone E2-7-7 was, in comparison, not active.

In parallel, studies were performed in the E2-10-20 and E2-7-7 on their ability to present antigen. The different clones were fed with KLH and were then treated with mitomycin C. Graded numbers of these cells were plated in 96 well, flat-bottomed microtiter plates. KLH-primed lymph node cells or nylon wool-purified T cells,[17] or T or B cell populations enriched by the panning technique[18] were added thereafter to the

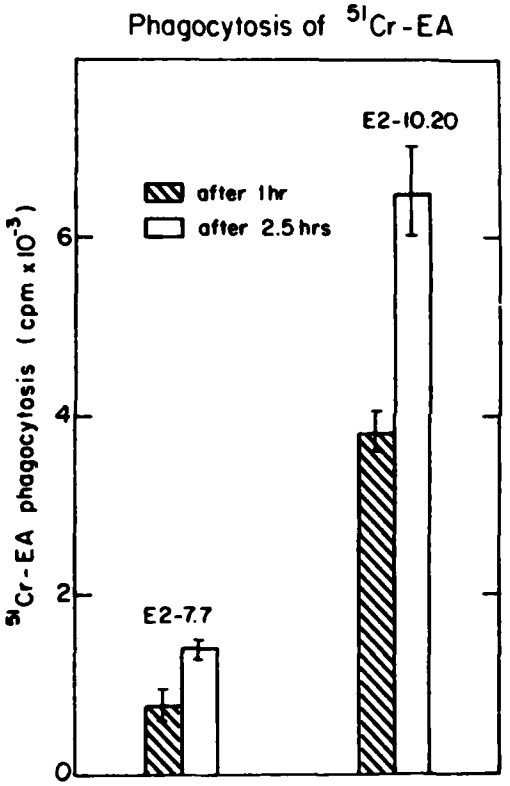

FIGURE 3. Phagocytosis by hybridoma clones E2-7-7 and E2-10-20. Target particles of opsonized and labeled sheep red blood cells were incubated with monolayers of clones for 1 hr and 2.5 hrs. Each bar represents quadruples with an SEM of less than 5%.

macrophage hybridoma monolayers. Control clones were treated in the same way without KLH. The proliferative responses to KLH of the restimulated cells was determined by [^{3}H] thymidine incorporation. The results indicate, FIGURE 4, that the E2-7-7 hybrid can present antigen to primed lymph node cells. Proliferation of lymph node cells, although antigen-specific, may result from a possible carry-over of antigen on the cell surface, and not the actual immunogenic capacity. We therefore tested the antigen presentation to primed T cells, enriched either by nylon wool filtration, or by the panning technique. It was shown that cells of the E2-7-7 macrophage hybridoma clone are potent in restimulating primed T cells. None of the clones presented antigen to primed B cells. It is conceivable that the observed variations in T-cell stimulation can be explained by differences in antigen uptake during pulsing. To exclude this possibility, the different macrophage hybridoma clones were tested for pinocytosis of ^{125}I-KLH, under conditions identical to those used for immunostimulation (TABLE 2).

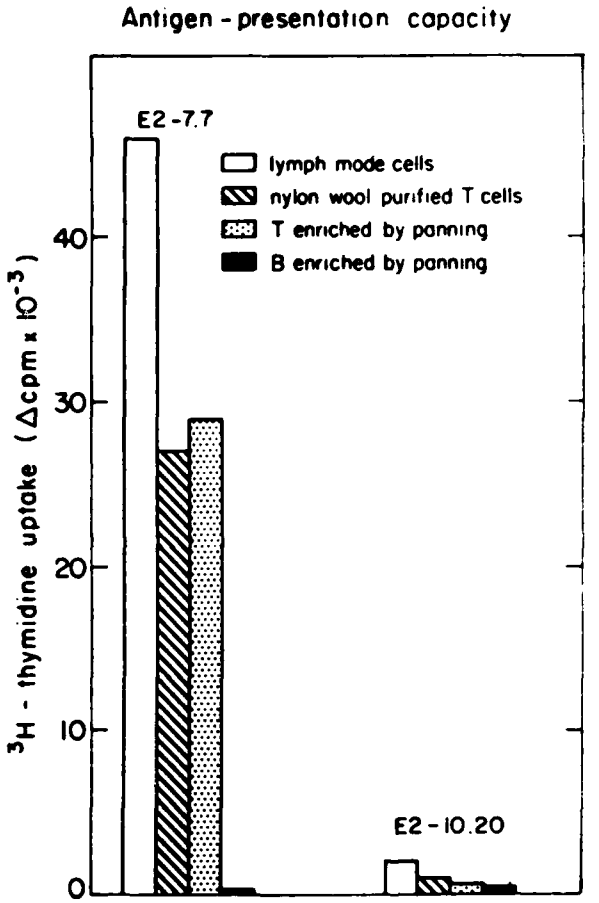

FIGURE 4. Antigen-presenting capacity of each clone. KLH was fed to clones that were then incubated with primed cells as indicated above.

The results indicated that the two clones showed comparable antigen uptake. No correlation was found between the amount of antigen taken up and the proliferative response of primed T cells.

DISCUSSION

We generated macrophage-myeloma cell hybridomas. The cells were initially screened for nonspecific esterase activity, lysozyme secretion, and tuftsin specific binding. Two hybrids contained the esterase activity and possessed the ability to bind tuftsin. Only the E2-7 could secrete lysozyme. Cloning and characterization of the above hybrids produced a series of clones, of which two were closely inspected. These

TABLE 2. ^{125}I-KLH Uptake by Different MØ-Hybridomas

Clone	^{125}I-KLH Uptake (cpm ± SD)[a]	After Trypsinization[b]
E2-7-7	186,868 ± 20,598	193,171 ± 12,213
E2-10-20	206,302 ± 10,872	216,722 ± 20,853

[a] 5×10^6 cells were incubated in presence of 50 μg/ml KLH laced with ^{125}I-KLH (10^7 cpm) for 90 min at 37°C.

[b] Following pinocytosis (as described above), the samples were trypsinized for 30 min and assayed for ^{125}I-KLH uptake.

clones, E2-7-7 and E2-10-20, are characterized by the existence of distinct surface markers and biological activity. The E2-7-7 macrophage-hybrid clone resembles macrophage morphologically, adheres to plastic, manifests esterase activity, binds tuftsin and secretes lysozyme. This clone is Ia$^+$, but does not possess Fc receptor. As to biological functions, E2-7-7 is very poor in phagocytosis, but manifests an antigen-presenting capacity to primed T cells. On the other hand, we could demonstrate esterase activity on the E2-10-20 clone, and binding of tuftsin. This clone was found to be Fc-receptor positive, but does not express Ia antigen on the cell surface. E2-10-20 is very efficient in phagocytosis, but could not present antigen to primed T cells.

We suggest that these two hybrids, E2-7-7 and E2-10-20 may represent different macrophage subpopulations, or at least different stages in differentation of the monocyte-macrophage lineage. However, we are aware of the fact that these clones may be segregants of the same cells that were obtained due to the hybridization process.

It is of interest that both clones that we characterized above are able to bind tuftsin. Both have binding affinities and binding-site quantities close to those established for thioglycollate-stimulated macrophages.[2,16] We suggest that the various activities attributed to the stimulatory affect of tuftsin in macrophages might be due to the effect of tuftsin in a particular subset of macrophage cells. Thus, the stimulatory effect of tuftsin on antigen presentation would be through macrophages containing the Ia alloantigen, while that of phagocytosis would be through macrophages that contain large quantities of the Fc receptor. Currently, we are studying the biological effect on these cells by tuftsin stimulation.

A corollary to the above hybridoma clones may exist for normal macrophages induced by thioglycollate. Tzehoval *et al.*[19] have separated distinct macrophage subclasses using a BSA discontinuous gradient. It was shown that one distinct macrophage subset contains the Ia alloantigen and presents to T cells. Another set is devoid of Ia alloantigen. Both sets are able to bind tuftsin (unpublished results). Whether these two macrophage types separated from normal macrophages correlate with the hybridomas we have isolated is currently under investigation.

The tuftsin sequence is found in the C_{H2} region of the Fc portion of IgG. This, coupled with its ability to bind to macrophage cells, has instigated the speculation that tuftsin binds to the Fc receptor of phagocytic cells. The generation of hybrid cells with diverse and distinct membrane markers that bind tuftsin indicates that no correlation exists between the existence of Fc receptor and the ability to bind tuftsin. A comparison of E2-10-20, which possesses significant Fc receptor quantities, and E2-2-7, which lacks this receptor while both retain the ability to bind tuftsin, verifies this (TABLE 1).

On the other hand, we have recently shown that the tuftsin receptor recognizes and binds specifically molecules that are related to the IgG sequence surrounding tuftsin (see tuftsin receptor, this edition). It thus appears that tuftsin may bind to a homologous but distinct receptor site from the Fc receptor. Unkeless[20] has recently demonstrated the existence of specific receptor sites for nonaggregated IgG, distinct from the receptor that binds the aggregated form. The number of sites and the affinity of binding is close to that found for tuftsin binding. Currently, we are investigating the possibility that tuftsin binds to such an Fc receptor.

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Receptor-mediated Internalization of Tuftsin[a]

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INTRODUCTION

One of the major problems in cell biology is the understanding of the mechanism b which extracellular ligands interact with their specific cell surface receptors t regulate intracellular metabolic events. It is now known that many ligands that bind t their specific receptors are rapidly internalized. This process, termed receptor-mediated endocytosis, has become recognized as the general mechanism by which cells acquire the necessary nutritional and regulatory proteins from their extracellular environment.[1-3] Some biologically important molecules known to be taken up by this mechanism include transport proteins such as low-density lipoprotein,[4] and transcobalamin II[5] as well as effector molecules such as insulin,[6-8] epidermal growth factor,[9] and the N-formyl chemotactic peptide.[10]

In many cases, rapid internalization of receptor-bound ligands is achieved through the clustering of receptors from an initial diffuse distribution. The total process may be accomplished within minutes at physiological temperatures, as is the case with the N-formyl chemotactic peptide bound to human granulocytes.[10]

In addition to having receptors for the N-formyl chemotactic peptide, human granulocytes are known to possess specific receptors for tuftsin, an immunopotentiating tetrapeptide of the sequence L-Thr-L-Lys-L-Pro-L-Arg.[11] Since the mechanism of action of tuftsin following binding to its receptor and the fate of the tuftsin molecule itself were not known, it was in our interest to delineate the early stages of tuftsin action following receptor occupancy. To accomplish this, a biologically active fluorescent analogue of tuftsin was prepared so we could visualize its interaction on human granulocytes and monocytes using video intensification microscopy (VIM). This study shows that tuftsin receptors are initially distributed diffusely and relatively homogeneously on the cell surface, then rapidly redistribute to form clusters that are then internalized.

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MATERIALS AND METHODS

Materials

Synthetic tuftsin, synthesized by the liquid phase method,[12] was obtained from Takeda Chemical Co., Osaka, Japan. Its amino acid composition was analyzed and was checked for purity by high-performance liquid chromatography (HPLC).[13] Sodium citrate buffer, constant-boiling HCl, ninhydrin, Pico Buffer System II, and threonine were all purchased from Pierce Chemical Co., Rockford, IL. Fluorescein isothiocyanate (FITC), was purchased from Research Organics, Cleveland, OH. SP-sephadex (C-25) was from Pharmacia, Piscataway, NJ, and Bio-Gel P2 from Bio-Rad Laboratories, Richmond, CA. Reagent-grade ethyl acetate was obtained from Fisher Scientific, Pittsburgh, PA, and trifluoroacetic acid (TFA) was obtained from Aldrich Chemical Co., Milwaukee, WI. All other chemicals were reagent grade unless specified otherwise. For cellular work, RPMI was purchased from Gibco, Grant Island, NY. Bovine serum albumin (BSA), was obtained from Miles Laboratories, Elkhart, IN and fetal calf serum (FCS) from Irvine Scientific, Santa Ana, CA. The water used was of type I grade with a resistance of 18 megaohms or better.

Fluorescent Labeling of Tuftsin

Fluorescein isothiocyanate (11.5 mg) was dissolved in 0.4 ml of 0.1 *N* KOH. To this solution, 1.6 ml of 0.1 *M* $NaHCO_3/Na_2CO_3$ coupling buffer, pH 8.3, was added. The resulting solution was added dropwise to a 1-ml solution of tuftsin (5 mg/ml) in coupling buffer. The pH was adjusted with 0.1 *N* HCl or NaOH to maintain a pH of 8.3. The reaction was allowed to proceed with constant mixing at room temperature for 3.5 hr. All handling of FITC-peptide was carried out in the dark.

Ion-Exchange and Gel Filtration Chromatography

Upon completion of the reaction, the pH of the solution was adjusted to 7.0 and applied to a column of SP-sephadex (1.5 × 20 cm) equilibrated with phosphate-buffered saline (PBS), pH 7.0. The flow rate was maintained at 30 ml/hr and 2-ml fractions were collected. Any remaining uncoupled tuftsin was eluted by raising the salt concentration in a stepwise manner. Free and coupled FITC was detected by absorbance at 495 nm, and the presence of peptide was detected at 570 nm using the ninhydrin reaction after hydrolysis.[14] Absorbance was monitored on a Hitachi model 100-20 spectrophotometer. Fractions absorbing at both wavelengths were concentrated and applied to a Bio-Gel P2 column (1.5 × 100 cm) and eluted with a 0.1 *M* $NH_4HCO_3/(NH_4)_2CO_3$ buffer, pH 8.5. The flow rate was maintained at 7.0 ml/hr. The effluent was monitored at 495 nm.

Reversed-Phase HPLC and Thin-Layer Chromatography

The columns used were an analytical and semipreparative μ-Bondapak C_{18} (Water Associates, Milford, MA), 3.9 mm × 20 cm and 7.8 mm × 30 cm, respectively; particle size was 10 μm. Columns were coupled to a model HPLPS-1 liquid pumping system (Glenco, Houston, TX).

Sample injections were made with a Glenco model SV-3 sample injection valve in

conjunction with Glenco VIS 100-μl and 500-μl syringes. All solvents were HPLC grade (Burdick and Jackson Labs, Muskegon, MI) and were filtered using a 0.2-μm filter (Fisher Scientific, Pittsburgh, PA) and degassed. Gradient elution was performed with a Glenco gradient mixer. A flow rate of 0.8 ml/min was maintained by a pressure of 1000 psi and detection was at 495 nm. All chromatographic runs were conducted at room temperature. One hundred micrograms in 100-μl and 200-μg samples in 500 μl of starting solvent were used for injections into analytical and semipreparative columns, respectively. Columns were equilibrated for at least 30 min with starting solvent.

Reversed-phase TLC was carried out in a solvent system of 60%/40% methanol/water on C_{18} plates (5 × 20 cm, Whatman, Inc., Clifton, NJ). Tenth molar NaCl was added to stabilize the binding to the plate.

Amino Acid Analysis

Samples were concentrated, dissolved in 500 μl of constant-boiling HCl and hydrolyzed *in vacuo* at 105°C for 18 hr. Samples were then washed three times with water, and dissolved in 500 μl of sodium citrate buffer (pH 2.2) before analysis. One-hundred-microliter samples were analyzed on a Glenco MM-60 single-column amino-acid analyzer, supplied with a cation exchange resin (HP-C, Bio-Rad Laboratories, Richmond, CA). Samples were eluted with sodium citrate buffers, (Pico Buffer system II) and monitored simultaneously at 440 and 570 nm using ninhydrin as the detecting reagent.

Cleavage of NH_2-terminal Amino Acids Coupled to FITC

Thirty to fifty nanomoles of HPLC-purified FITC-tuftsin were treated with 200 μl of TFA that had been saturated with HCl gas. The solution was allowed to stand at room temperature for 2 hr. The acid was then removed *in vacuo* over KOH. The dried residue was then treated with 200 μl of 0.5 *N* HCl for 15 hr followed by extraction with ethyl acetate. The ethyl acetate extract was then subjected to TLC on silica gel G plates (5 × 20 cm, Analtech, Newark, DE) in a solvent system consisting of chloroform : pyridine : acetic acid (10:1:2 by volume).[15,16] The FITC-threonine standard used was synthesized and purified, and underwent the same NH_2-terminal cleavage conditions just described.

Dansylation

Dansylation of fluoresceinated tuftsin was carried out according to the method of Gray.[17] Five-nanomole samples were used and the reaction was allowed to proceed at room temperature for 3 hr.

Preparation of Cells

Human granulocytes were prepared according to the method of Chee *et al.*[18] The monocytes from the mononuclear cell fraction were isolated by their characteristic adherence to plastic.

Biological Activity Assay

The assay used was that described by Phillips *et al.*[19] Purified human polymorphonuclear leukocytes (PMN) were incubated with *Staphyloccus aureus* with or without tuftsin or FITC-tuftsin, for 10 min at 37°C at the following concentrations: 0.1, 0.2, 0.4, 0.6, 1.0 μg/ml. Following this incubation, the cells were washed, a 400-μl aliquot was removed (t = 0), and the remainder returned to 37°C for 3 hr. At this time, a second aliquot was removed (t = 3). The aliquots were sonicated, plated in microtiter trays containing trypticase soy broth from which dilutions were made to a final titer of 10^{-7}. They were incubated for 16 hr at 37°C and each well scored as either positive or negative for growth. From the number of viable bacteria calculated, the following ratios were determined: phagocytosis index = NTt = 0/NCt = 0; bactericidal index = (NTt = 3/NTt = 0)/(NCt = 3/NCt = 0) where NTt = 0 and NTt = 3 are the numbers of viable bacteria ingested by tuftsin-treated PMN after 10 min and 3 hr, respectively, and NCt = 0 and NCt = 3 are the numbers of viable bacteria ingested by nontuftsin-treated PMN after 10 min and 3 hr, respectively.

Binding of FITC-Tuftsin to Human PMN and Monocytes

Purified monocytes or PMN were washed and allowed to adhere to glass slides for 5 min at 37°C in PBS containing 2% BSA. The FITC-tuftsin was layered onto the slides to a final concentration of 2.6×10^{-7} *M* and incubated at 37°C for various times. Before observation, the slides were quickly rinsed in 37°C buffer. For dual-labeled studies, heat-aggregated IgG was labeled with tetramethyl-rhodamine isothiocyanate (Research Organics, Cleveland, OH) according to the method of Arbeit *et al.*[20] Rhodamine-labeled aggregated IgG (R – IgG) (2 mg/ml) was added to PMN simultaneously with FITC-tuftsin (2.6×10^{-7} *M*) for 5 min at 37°C or alone for 10 min followed by addition of FITC-tuftsin for 5 min at 37°C.

Video Intensification Microscopy

Binding of FITC-tuftsin to PMN and monocytes was observed by means of an RCA silicon intensifier target TV camera (TC1030H) attached to a Leitz Diavert inverted fluorescent microscope. The enclosed microscope stage was monitored at 37°C using a Thermistep Temperature Control (Model 71A, Yellow Springs Instrument Co., Yellow Springs, OH). Video output was recorded on a Panasonic time-lapse video tape recorder (NV-8030) and displayed on a Panasonic TV monitor (WV-5300), from which polaroid photographs were taken.[21] Lenses include a Zeiss Neofluar 100X, 1.3 numerical aperature, a Zeiss Planapo 63X, 1.4 numerical aperature, and a Zeiss Planapo 40X, 1.0 numerical aperature.

RESULTS

Purification of FITC-Tuftsin

It can be seen from FIGURE 1 that free tuftsin was separated from the conjugate by ion-exchange chromatography using a stepwise elution. Fractions absorbing both at 495 and 570 nm were individually chromatographed on a Bio-Gel P2 column as

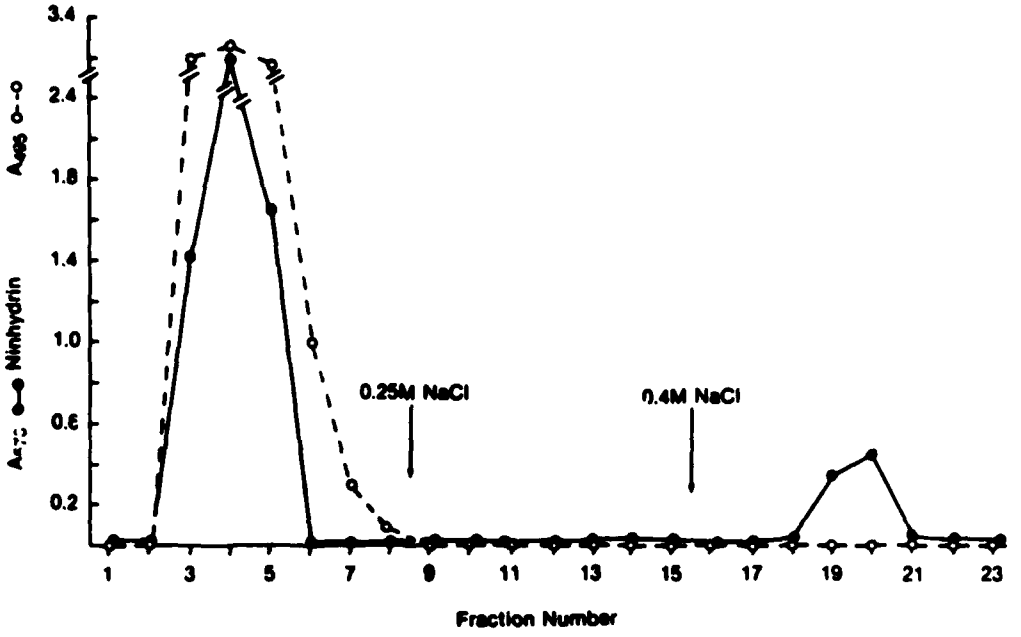


FIGURE 1. Separation of free tuftsin from free FITC and FITC-tuftsin on an SP-Sephadex C-25 ion-exchange column (1.5 × 20 cm). The mobile phase consisted of PBS, pH 7.0. The flow rate was 30 ml/hr. Two-milliliter fractions were collected.

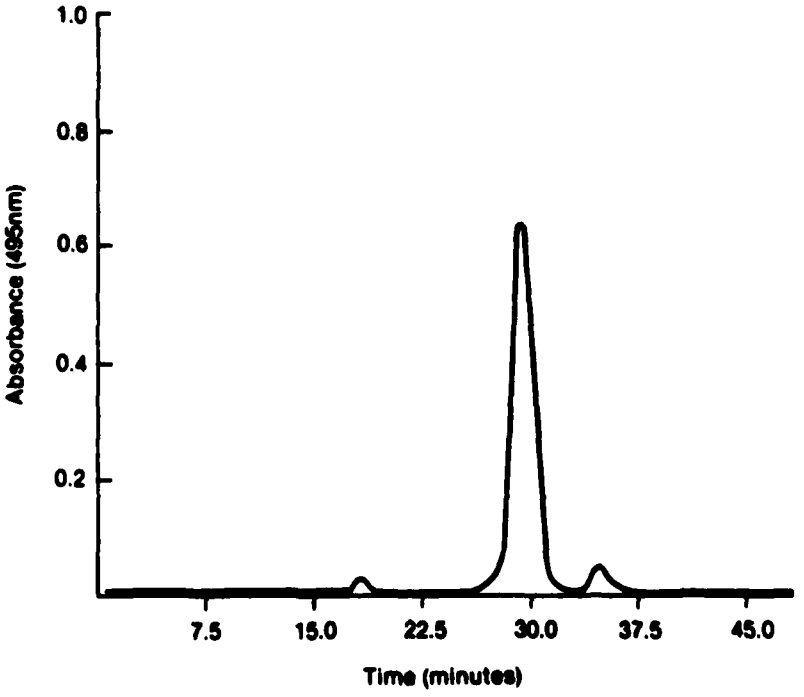


FIGURE 2. Reversed-phase HPLC of FITC-tuftsin on a μ-Bondapak C_{18} column (3.9 × 30 cm). The mobile phase, maintained at a flow rate of 0.8 ml/min, consisted of a 20%/60% methanol:water linear gradient (40 ml total volume). One-milliliter fractions were collected. A sample of 100 μg in 100 μl was applied. Fractions from the leading half of the major peak were pooled and used throughout the remainder of the study.

TABLE 1. Amino Acid Analysis Ratios of Tuftsin and FITC-Tuftsin

	Thr	Lys	Pro	Arg
Tuftsin	0.90	0.96	1.02	1
FITC-Tuftsin	0.25	0.90	0.92	1
	0.18	0.91	1.04	1
	0.16	0.94	0.95	1
	0.26	0.95	1.01	1

described in MATERIALS AND METHODS. This purification step was used to separate free FITC from the conjugate. Detection was at 495 nm for the presence of FITC and by amino acid analysis for the presence of peptide.

Reversed-phase HPLC was employed to remove any isomers conjugated to tuftsin, since isomers of FITC are present in most commercial preparations of the fluorochrome. The profile of FITC-tuftsin is shown in FIGURE 2. All three peaks were positive for tuftsin as judged by amino acid analysis. Five microliters from individual fractions of the major peak were spotted on reversed-phase TLC plates and chromatographed using the conditions described in MATERIALS AND METHODS. The results (not shown) showed a major spot (Rf 0.65) for the leading half of the major peak but an additional minor spot (Rf 0.60) for the trailing half. Subsequently, the homogeneous leading portion of the major peak was collected and used throughout the remainder of the study. Since the TLC plates have a higher percent carbon load and surface coverage of the C_{18}-bonded phase than the column used, this may account for their ability to resolve the second component.

Characterization of the Coupling Site

Evidence of a modified α-amino terminal is given in TABLE 1 where the results of four separate hydrolyses and analyses of FITC-tuftsin are shown. In two cases, there is approximately an 80% decrease in the threonine ratio whereas the other two show a 70% decrease. This is to be expected if the NH_2-terminus is coupled since loss of the positive charge would shift threonine's time and position of elution from the ion-exchange column. Since free tuftsin is not retained on a C_{18} reversed-phase column in the presence of organic solvents,[13] the presence of free tuftsin in the sample can be ruled out as the cause for the small amount of threonine remaining, as judged by amino acid analysis. This is most likely due to cleavage of a small percentage of thiocarbamyl bonds during hydrolysis. Two other experiments were undertaken to confirm derivatization of the α-amino group. First, cleavage of the NH_2-terminal amino acid yielded only free FITC-threonine upon analysis by TLC (FIG. 3). The major component migrated with an Rf of 0.30.

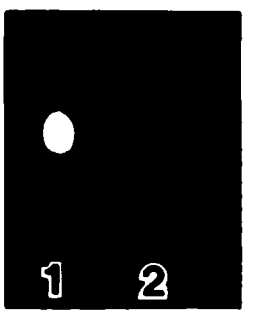


FIGURE 3. TLC of the ethyl acetate extract after NH_2-terminal cleavage of FITC-tuftsin (lane 2) and FITC-threonine standard (lane 1). The solvent system consisted of chloroform:pyridine:acetic acid (10:1:2) and chromatography was performed on a silica gel G plate.

TABLE 2. Amino Acid Analysis Ratios of Dansylated FITC-Tuftsin

Thr	Lys	Pro	Arg
0.21	0.29	1	0.92
0.30	0.22	1	0.95
0.27	0.31	1	0.97

The second experiment entailed dansylation of the FITC-tuftsin to determine if the ϵ-amino group of lysine is indeed free (TABLE 2). Incomplete derivitization is most likely due to cleavage of a small percentage of dansyl groups during hydrolysis. The conclusion of a mono-fluoresceinated tuftsin molecule modified at the α-amino terminal is substantiated. Also, since FITC reacts with unprotonated amino groups, the only unprotonated amino group at the coupling pH chosen exists at the α-amino terminus, as can be concluded from the respective pk values of the tuftsin functional groups (pk α-NH_2, 7.1; pk ϵ-NH_2, 10.0).[22]

Biological Activity of FITC-Tuftsin

FIGURE 4 compares the phagocytic-enhancing ability of fluorescent tuftsin and tuftsin at five different concentrations. At each concentration, a different blood donor was employed for the isolated PMN used in the assay and in each case, FITC-tuftsin, tuftsin, and controls were tested simultaneously. In this case, values greater than unity represent enhancement of phagocytosis. As can be seen from the graph, there is no significant difference in the phagocytic indicies between FITC-tuftsin and tuftsin.

FIGURE 5 compares the bactericidal indices of FITC-tuftsin and tuftsin. In this case, values less than unity represent enhancement of the bactericidal activity. Again, as can be seen from the graph, there is no significant difference in the bactericidal indices between fluoresceinated tuftsin and tuftsin.

Binding and Redistribution of FITC-Tuftsin on Human PMN and Monocytes as Demonstrated by VIM

Polymorphonuclear leukocytes that were exposed to FITC-tuftsin for 1 min at 37°C displayed a relatively homogeneous and diffuse membrane fluorescence pattern

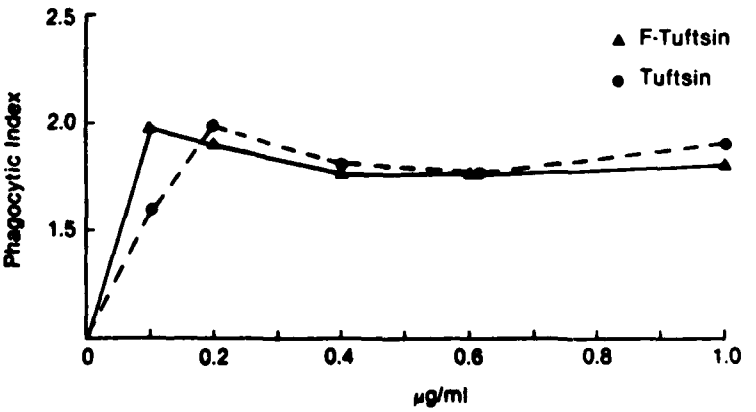


FIGURE 4. Phagocytosis index = NTt = 0/NCt = 0, where NTt = 0 and NCt = 0 are the number of viable bacteria ingested by tuftsin-treated and control PMN after 10 min, respectively.

(FIG. 6a). By 3 min some patching begins to occur; however, a diffuse background can still be seen (FIG. 6b). These patches were confined to the membrane at this time since they were contained only in the focal plane of the membrane. By 6 min, distinct aggregates of intense fluorescence could be seen (FIG. 6c). Some aggregates had already been internalized by this time, as evidenced by their saltatory motion. By 10 min, coalescence of membrane patches and internalization continued, so that only 5–10 patches remained on the cell surface (FIG. 6d).

When PMN were exposed to fluoresceinated tuftsin (2.6×10^{-7} *M*) and nonfluoresceinated tuftsin (2.6×10^{-5} *M*), membrane staining, aggregation, and internalization of fluorescent material was not seen (FIG. 7a and b). Free tuftsin and free FITC added simultaneously showed no cell-associated fluorescence. When binding was carried out at 4°C for 20 min, the homogeneous, diffuse membrane fluorescent pattern was similar to that in FIG. 6a. (FIG. 7c). Upon warming to 37°C, aggregation and internalization of fluorescent aggregates ensued.

When fluorescein-labeled tuftsin (2.6×10^{-7} *M*) and R-IgG (2 mg/ml) were added simultaneously to human PMN and incubated for 5 min at 37°C, a diffuse

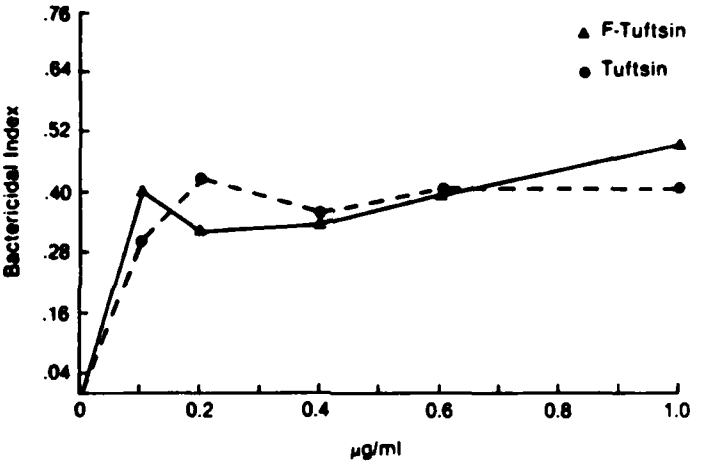


FIGURE 5. Bactericidal index = (NTt = 3/NTt = 0)/(NCt = 3/NCt = 0), where NTt = 0 and NTt = 3 are the numbers of viable bacteria ingested by tuftsin-treated PMN after 10 min and 3 hr, respectively, and NCt = 0 and NCt = 3 are the numbers of viable bacteria ingested by nontuftsin-treated PMN after 10 min and 3 hr, respectively.

pattern was observed with the R-IgG (FIG. 8a) but distinct aggregates were already formed with FITC-tuftsin (FIG. 8b). When R-IgG was added first to human PMN (2 mg/ml) and incubated for 10 min at 37°C followed by addition of FITC-tuftsin (2.6×10^{-7} *M*) for 5 min, a typical capping phenomenon was observed with the R-IgG (FIG. 8c). When the cell was observed on the fluorescein channel, some comigration with FITC-tuftsin was evident; however, distinct patches of FITC-tuftsin also formed in areas where R-IgG was not present (FIG. 8d). If either ligand was added in excess, no competition was observed.

Binding of FITC-tuftsin to human peripheral blood monocytes showed similar patterns to those observed on human PMN. At 1 min and 37°C, a diffuse and homogeneous membrane fluorescene pattern was observed (FIG. 9a). By 8 min (FIG. 9b), redistribution into coalesced patches had occurred, and some internalization had already taken place as judged by saltation of fluorescent aggregates. By 15 min, only a few patches remained on the cell surface, most having become internalized by this time (FIG. 9c). An average percentage of monocytes binding FITC-tuftsin was 53 ± 7%

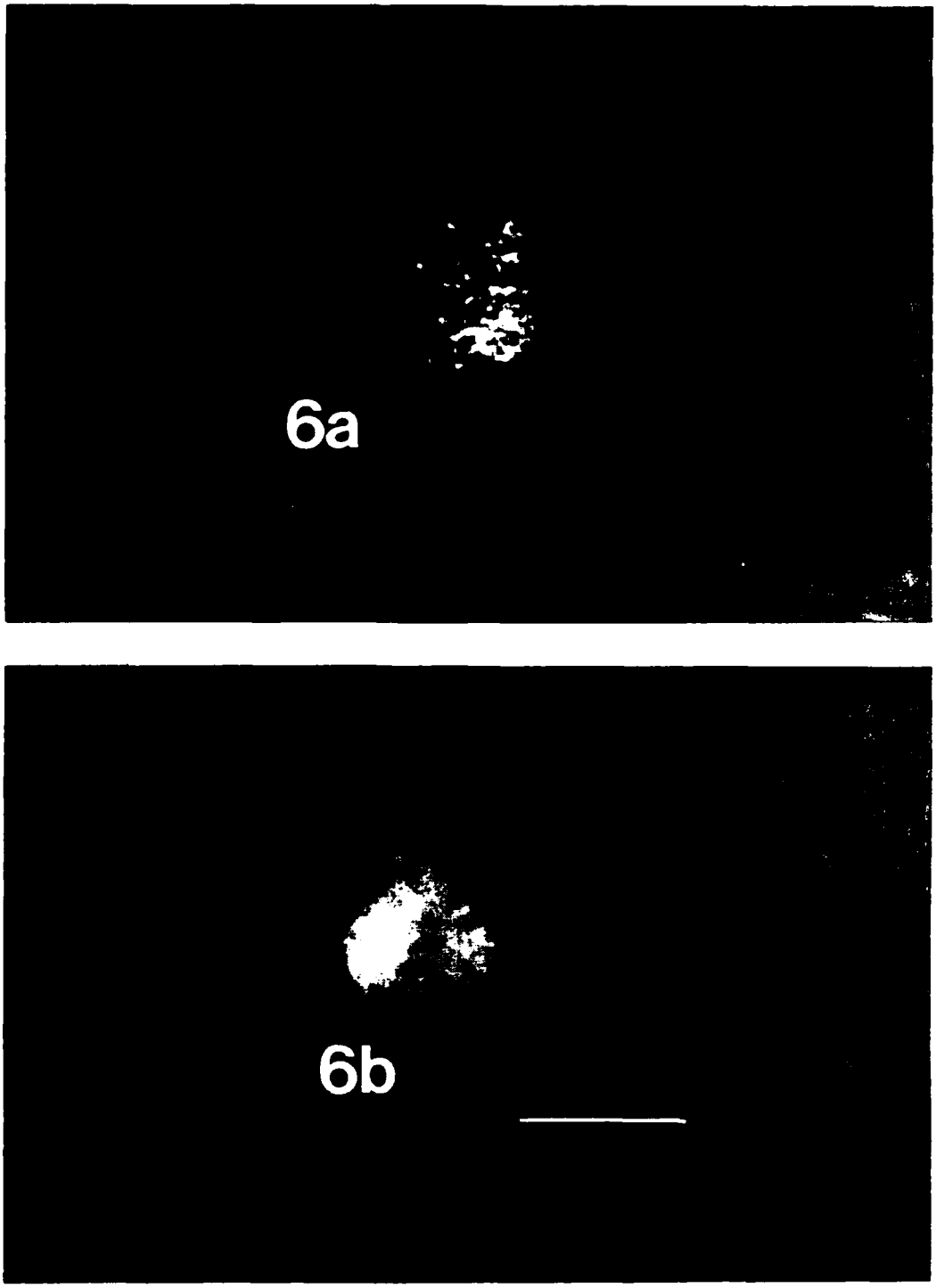
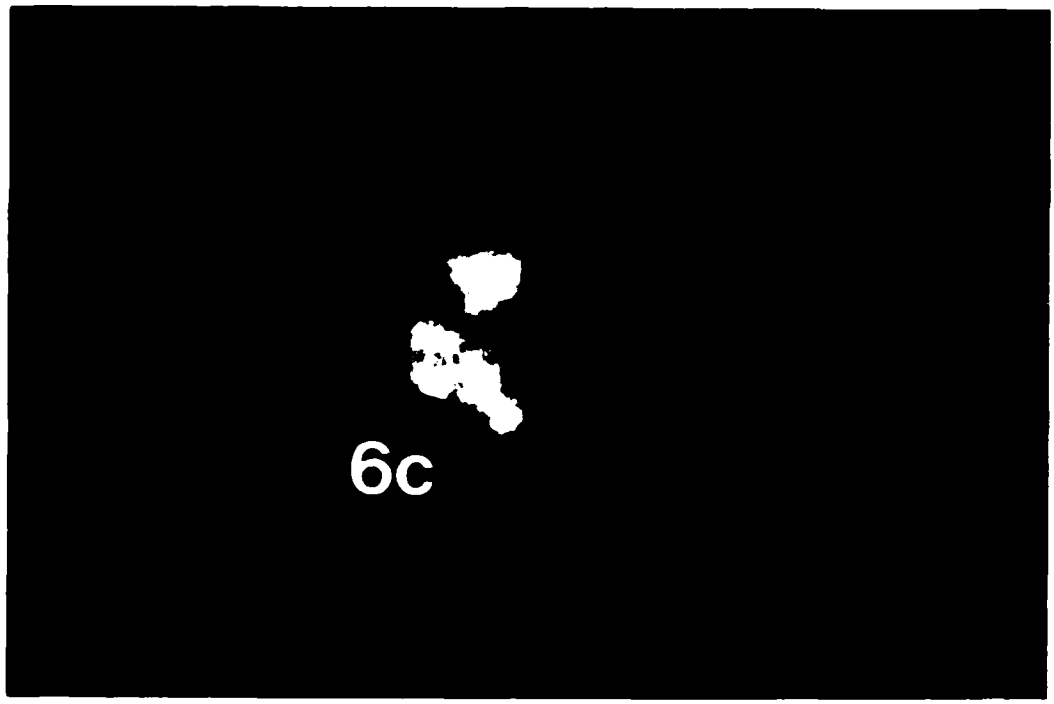


FIGURE 6. Binding of FITC-tuftsin of human PMN. Purified PMN were allowed to adhere to slides for 5 minutes at 37°C in PBS with 2% BSA. The fluorescent tuftsin was layered onto the slides to a final concentration of 2.6×10^{-7} *M* and incubated at 37°C in humidified air containing 5% CO_2. Cells were observed through an oil-immersion Planapo 63/1.4 numerical aperture lens (a and b) or a Planapo 40/1.0 numerical aperture lens (c and d) by fluorescence microscopy directly. Magnification on the video monitor was ×2000 for (a) and (b) (bar, 10 μm) and ×1270 for (c) and (d) (bar, 10 μm). The incubation times for fluorescent tuftsin were as follows: a, 1 min; b, 3 *min*; c, 6 *min*; d, 10 *min*.



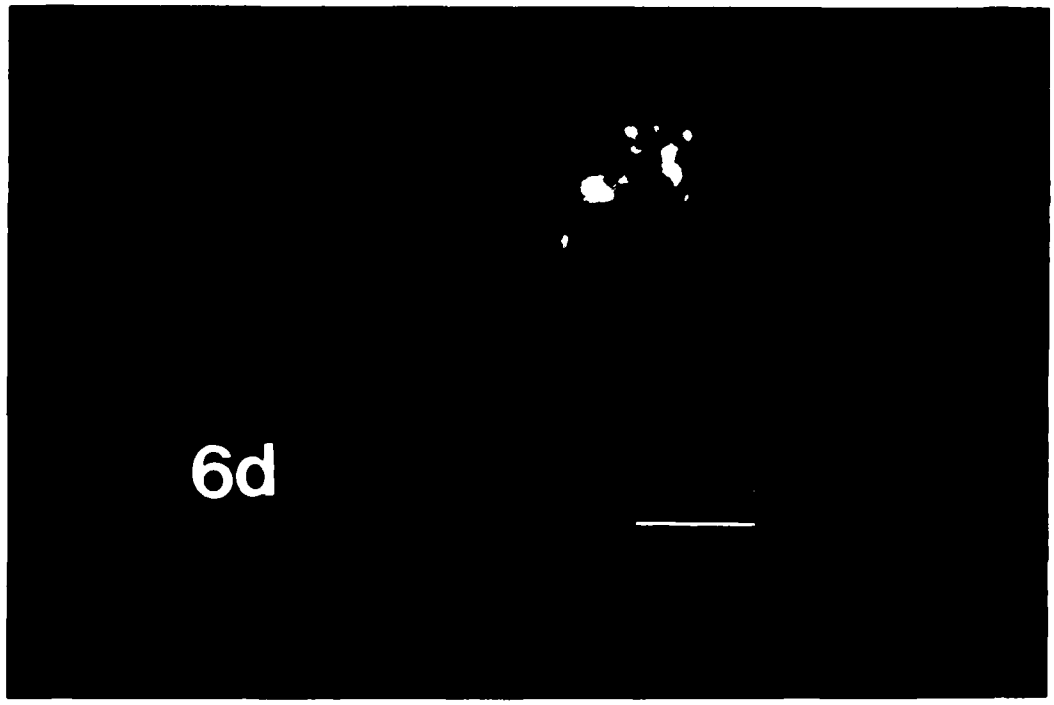


FIGURE 6. (Continued)

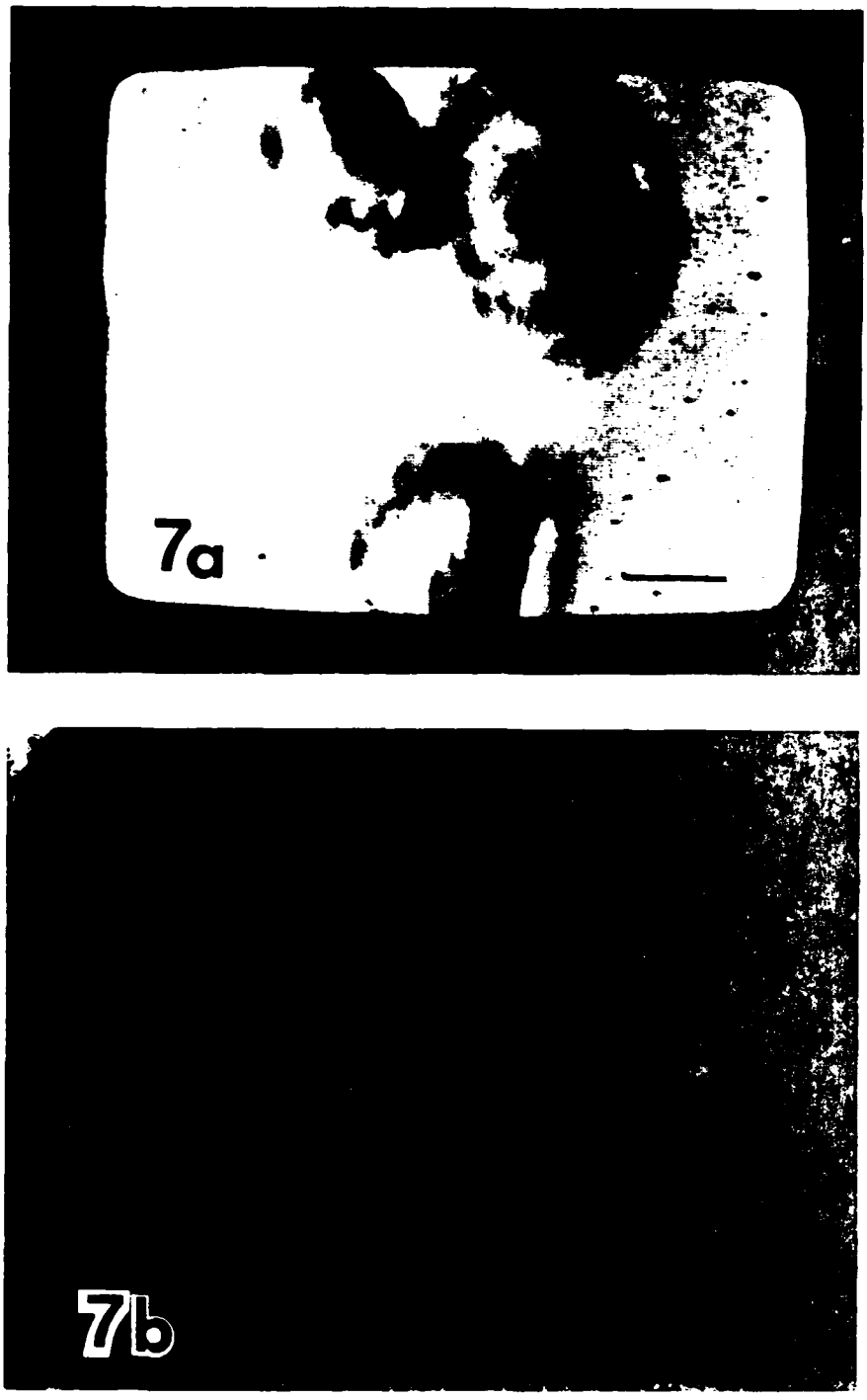


FIGURE 7. Specificity of binding was assessed by stimultaneous exposure of cells to FITC-tuftsin (2.6×10^{-7} *M*) and unlabeled tuftsin (2.6×10^{-5} *M*) for 3 min at 37°C. A Planapo 63/1.4 numerical aperture oil-immersion lens was used for phase contrast (a) and fluorescence microscopy (b). Magnification on the video monitor was ×1270, (bar, 10 μm). Binding of FITC-tuftsin to human PMN at 4°C. Purified PMN were allowed to adhere to slides for 5 min at 37°C in PBS with 2% BSA. Slides were then cooled at 4°C for 40 min. Fluorescent tuftsin was layered onto the slides to a final concentration of 2.6×10^{-7} *M* and incubated at 4°C for 20 min. Cells were observed through an oil-immersion Planapo 63/1.4 numerical aperature lens by fluorescence microscopy (c). Magnification on the video monitor was ×2000; (bar 10 μm).

(N = 10). This value was lower in the case of PMN, 43 ± 9% (N = 10). Preliminary evidence indicated that highly enriched NK cells also bind this analogue, but purified T cells, partially purified B cells, red blood cells, and platelets do not.

Internalization and Saltatory Motion as Demonstrated by VIM

Organelles are known to move about within cells by a process termed saltatory motion.[23] FIGURE 10 represents an experiment in which human PMN were incubated with FITC-tuftsin for 20 min at 37°C and then observed under high magnification for saltation. The position of a single internalized aggregate relative to a fixed point on the photograph at 0, 8, 15, and 21 sec is shown. It does not move steadily but in a saltatory

FIGURE 7. (Continued)

manner. Numerous saltations did occur during the sequence, but only representative positions are shown at the above time points. When cytoplasmic streaming was observed under phase contrast, the fluorescent aggregates moved in the same direction and with the same velocity as the cytoplasmic organelles. This substantiates the occurrence of internalization.

DISCUSSION

In this study, fluoresceinated tuftsin and VIM were used to delineate the membrane-associated macromolecular events that occurred after receptor occupancy. Direct fluorescent labeling offers advantages over indirect methods, especially when a

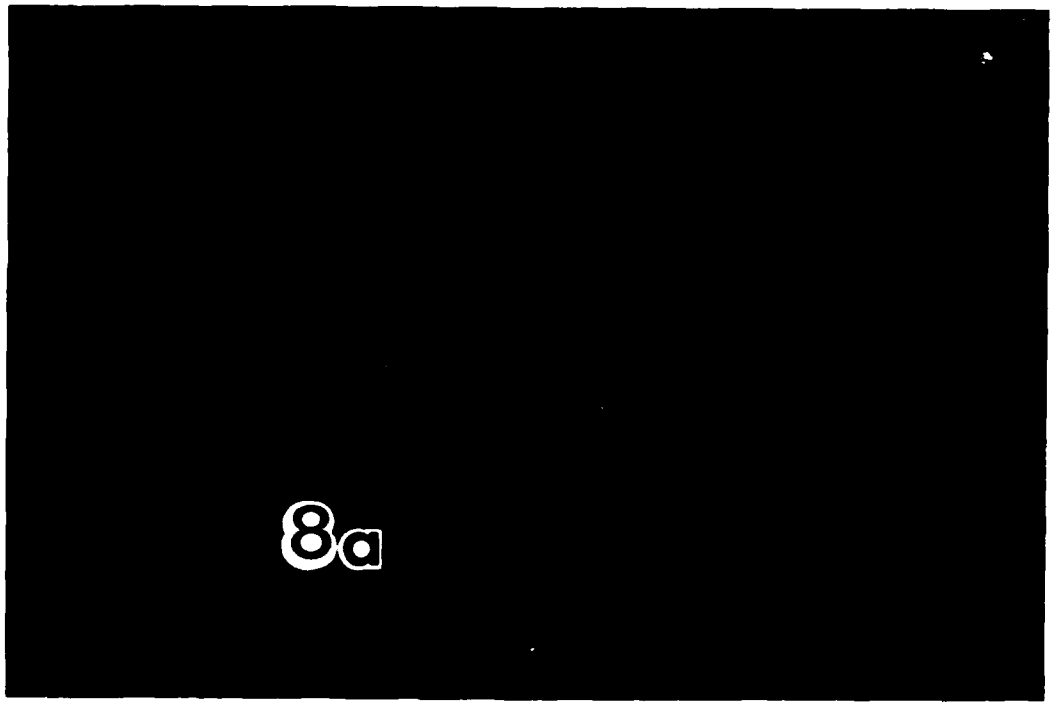


FIGURE 8. Binding of FITC-tuftsin and R-Ag-IgG to human PMN. Purified PMN were allowed to adhere to slides for 5 min at 37°C in PBS with 2% BSA. Fluorescent tuftsin (2.6×10^{-7} *M*) and R-Ag-IgG (2 mg/ml) were added simultaneously, incubated for 5 min at 37°C, rinsed and visualized for rhodamine (a) and fluorescein (b) fluorescence. In another study, R-Ag-IgG was added first for 10 min at 37°C, followed by the addition of FITC-tuftsin for 5 min. Again, the cells were visualized for rhodamine (c) and fluorescein (d) fluorescence. Magnification ×1270, bar, 10 μm.

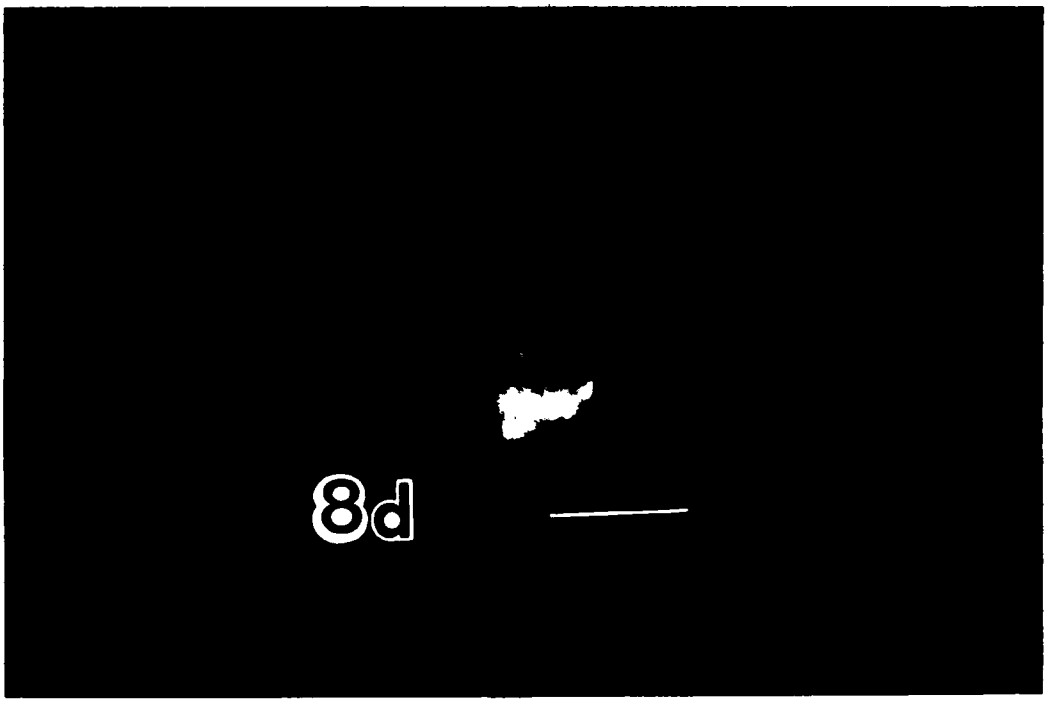


FIGURE 8. (Continued)

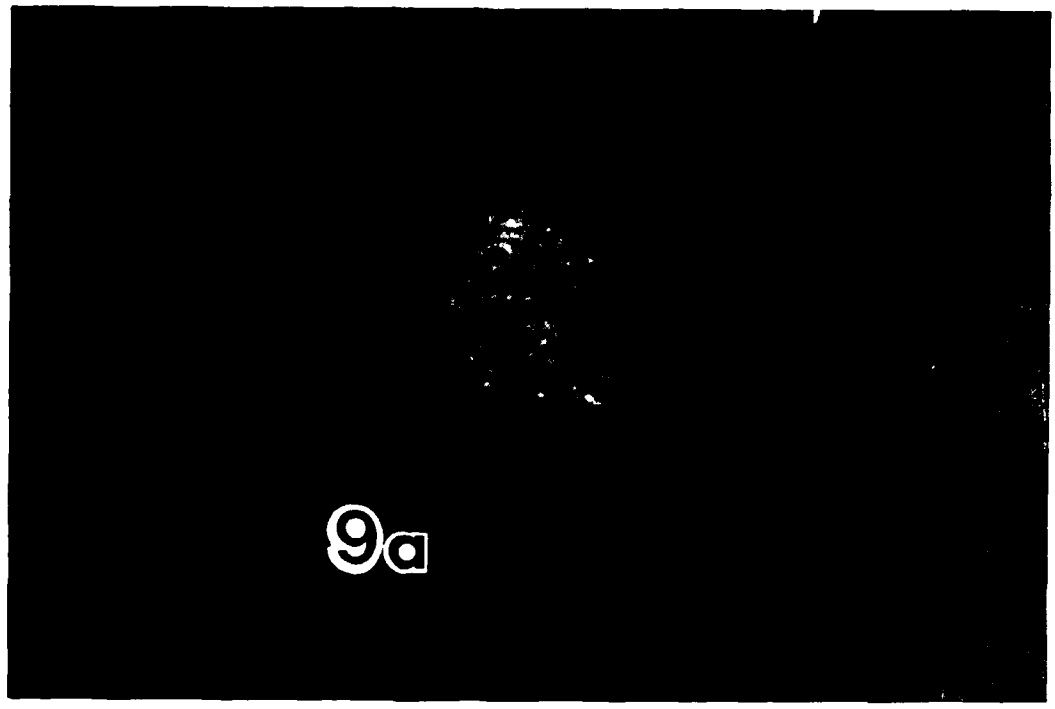


FIGURE 9. Binding of FITC-tuftsin to human monocytes. Purified monocytes were allowed to adhere to slides for 5 min at 37°C in PBS with 2% BSA. Fluorescent tuftsin was layered onto the slides to a final concentration of 2.6×10^{-7} *M*, incubated for various times, rinsed and visualized. Time points: a, 1 min; b, 8 min; c, 15 min. Magnification ×2000; bar, 10 μm.

small ligand such as tuftsin is involved. They include the ability to purify and characterize the analogue as well as to obtain a more realistic interpretation of the fate of the ligand. Characterization of the fluorescent analogue yielded a monofluoresceinated derivative, modified at the α-amino terminal. This analogue retained both the phagocytic- and bactericidal-stimulating abilities of the native molecule.

Another important advantage of this type of labeling is that it provides information of the structure-function relationship of the molecule. Preservation of the positive charge on the arginine side chain appears to be critical in retaining its biological activity.[24] Preservation of an intact NH_2-terminal has also been reported as critical in retaining tuftsin's phagocytosis-stimulating ability.[24] However, some analogues in which the NH_2-terminal had been cyclized were reported to possess considerable, if not all of, the biological activity.[25,26]

FIGURE 9. (Continued)

This fluorescent analogue enabled us to ask several questions about the early events of tuftsin's action that could not be answered with a radiolabeled ligand. The initial distribution of tuftsin receptors on the cell surface of PMN and monocytes was found to be diffuse and homogeneous. The ensuing events were comprised of rapid redistribution into patches, followed by internalization. The events were temperature dependent and the redistribution patterns were similar to those of N-formyl chemotactic hexapeptide,[10,27] and other proteins such as insulin,[28,29] epidermal growth factor,[30,31] and α_2-macroglobulin.[32,33] These patterns are dissimilar to low-density lipoprotein receptor distribution patterns, where unoccupied receptors have been reported to be clustered in coated pits.[34,35]

Aggregation or clustering of ligand-receptor complexes has been suggested as an important event in signal transduction. Immunoglobulins and mitogens, unlike some

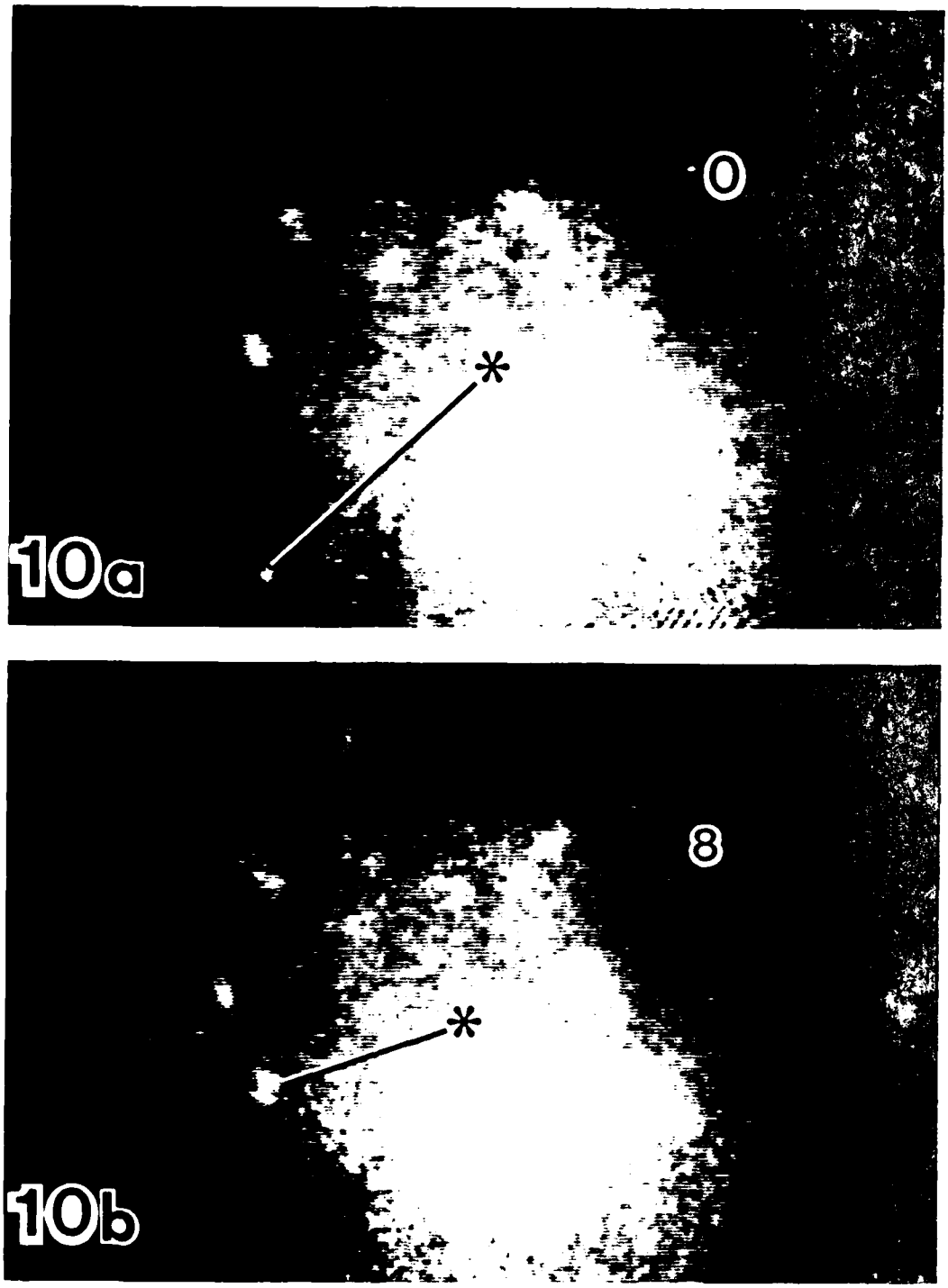


FIGURE 10. Saltatory motion of fluorescent aggregates after incubation with FITC tuftsin. PMN, after adhering to slides for 5 min at 37°C in PBS containing 2% BSA, were incubated with $2.6 \times 10^{-7} M$ α-FITC-tuftsin for 20 min at 37°C and observed under oil immersion using a Neofluar 100/1.3 numerical aperture lens by fluorescence microscopy. A time-lapse video tape recording at a 9:1 time-lapse ratio was made of a cell with intracytoplasmic saltatory motion of fluorescent aggregates. The numbers in the upper right-hand corner of each photograph (a and b) represent the real time in seconds after the start of the sequence. The asterisk represents an arbitrary nonmoving reference point. A line is drawn in each photograph from this point to a single fluorescent aggregate. Magnification on the video monitor was ×3170. (bar, 10 μm)

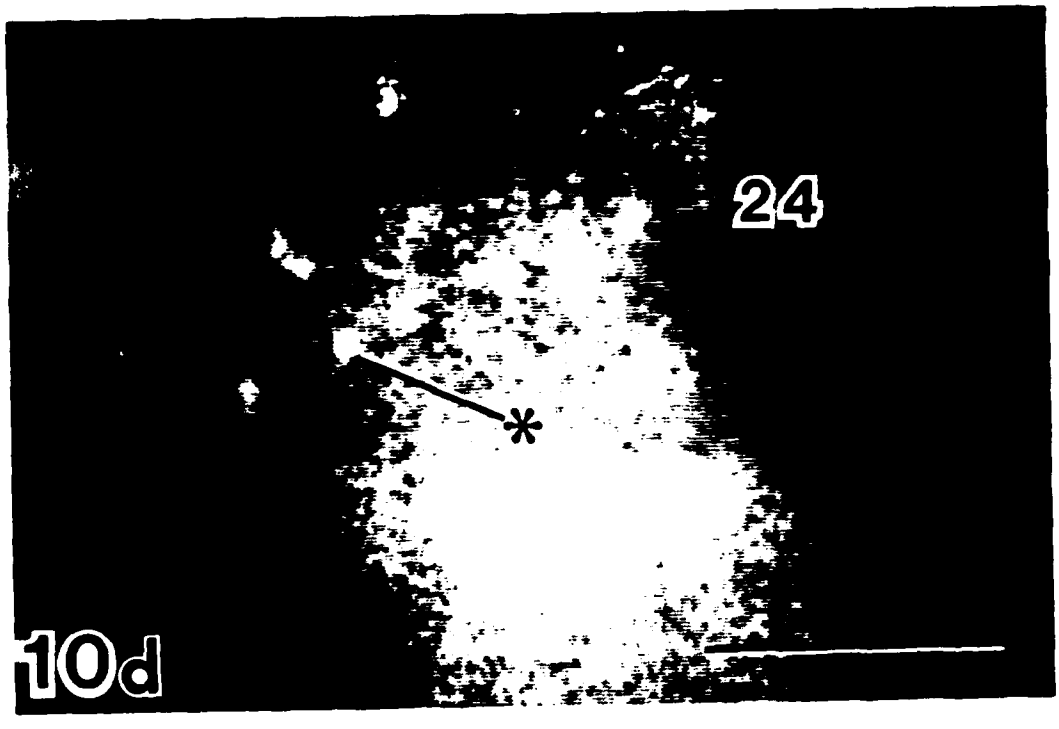


FIGURE 10. (Continued)

protein hormones, require a multivalent form of the ligand to effect redistribution on the cell surface. Tuftsin, like the chemotactic hexapeptide, accomplishes this in a monovalent state. This may reflect the way in which small peptides are dealt with by particular cell types.

Since tuftsin originates from the CH_2 domain of IgG, it has been suggested that it may bind to cell types bearing Fc receptors. However, the evidence presented in this study indicates there is no competition for binding between FITC-tuftsin and aggregated R-IgG. In addition, these ligands were handled differently on the cell surface as judged by their speed and pattern of redistribution. The fact that only a certain percentage of PMN and monocytes bound FITC-tuftsin raises the question of whether a certain subpopulation of cells express the tuftsin receptor on their surface. A more likely possibility is that some cells may have been exposed to endogenous tuftsin, which when internalized (presumably with its receptor), resulted in a so-called down regulation of the tuftsin receptor.

The membrane-associated macromolecular events delineated through the use of fluoresceinated tuftsin may be relevant to some of tuftsin's biological activities. Aggregation of tuftsin-receptor complexes into groups of two or more may be responsible for the transduction of certain signals. Since the working concentration of fluorescent tuftsin used was below the value reported for saturation, full occupancy must not be required for redistribution to occur.

The role of internalization may also have some relevance. Tuftsin, possibly with its receptor, may be able to associate with various organelles through internalization, which may be necessary to effect its biological response. Association of internalized aggregates with subcellular structures could not be determined accurately due to the limits of resolution of the VIM system as well as the characteristic bleaching of fluorescein. Whether the internalization of tuftsin is simply an intermediary event leading ultimately to degradation, or a crucial event in eliciting a biological response, it still may provide the target cells with an early time point in modulating its biological effect through down regulation of its receptors. This analogue will facilitate binding experiments, particularly with cells that cannot be obtained in pure form for radioligand studies.

ACKNOWLEDGMENTS

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The Similarity between Tuftsin (Thr-Lys-Pro-Arg) Receptors and Tuftsin Antibody: A Case of Induced Molecular Mimicry[a]

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INTRODUCTION

In 1947, Zamecnik and Lipman[1] showed that lecithin prevented the inhibition of *Cl. welchii* lecithinase activity by horse antibody to the enzyme. A similar finding was reported a year later by Krebs and Najjar who showed that the inhibition of yeast 3-phosphoglyceraldehyde dehydrogenase by rabbit antibody was counteracted by its substrate NAD.[2] Consequently, it was deduced by both groups that the anti-enzyme antibody reacted at the active site of the enzyme. The inhibition of the antibody reaction by substrate indicated that the antibody surface, in its physical topography, was patterned to fit the enzyme site in the manner of the substrate. On this basis, in 1951 it was postulated by Najjar[3] that if a second antibody could be induced against the reactive site of the anti-enzyme antibody (idiotype), the second anti-antibody (anti-idiotypic) would be similarly patterned as to mimic the enzyme surface and perhaps exhibit enzyme-like properties.[3] This appeared to be the case. The anti-idiotypic antibody generated in the rabbit against the horse anti-lecithinase seemed to acquire lecithin binding properties. The effect was not found in normal sera or antisera to other antigens. FIGURE 1 is a crude representative model of the proposed anti-antibody now known as anti-idiotypic antibody.

Recently, we were able to demonstrate a novel case of induced molecular mimicry involving antibody to the tetrapeptide tuftsin (Thr-Lys-Pro-Arg) and its specific receptors that are found on macrophages and granulocytes.[4,5] The binding properties of tuftsin receptors and tuftsin antibody proved to be similar with respect to six oligopeptides. Thr-Lys-Pro, Ala-Lys-tuftsin, Ala-Lys-tuftsin-Glu-Ala$_3$, [Glu]^3-tuftsin, [Pro-Pro^3]-tuftsin, and tuftsinyltuftsin. Peptides with no biological effect showed weak or no binding. By contrast, those that were biologically inhibitory showed stronger

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binding than tuftsin.[5] Our interest in tuftsin stems from the finding that it is a natural activator of phagocytic cells including the macrophage. In that capacity, it stimulates all the functions of the phagocyte; motility, phagocytosis,[6,7] immunogenic stimulation,[8,9] bactericidal activity[10] and tumoricidal activity.[5,9,11-13] Several human cases of tuftsin deficiency have been described in this country and Japan.[14] It also effects a considerable stimulation of age-depressed T-cell cytotoxic activity and age-depressed, tumoricidal activity of peritoneal macrophages.[15] These effects have since been reviewed.[12,16-19]

The purpose of this communication is to document in detail the similarity in binding characteristics between the membrane receptors of tuftsin and the active site of anti-tuftsin antibody.

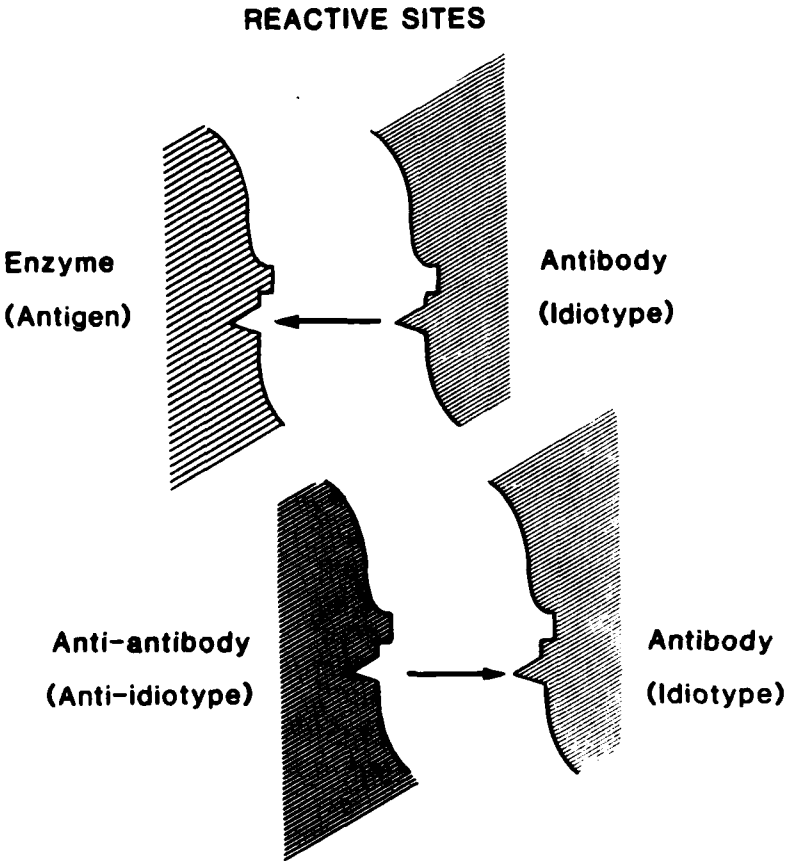

FIGURE 1. Representative model of anti-idiotypic antibody.

MATERIALS AND METHODS

The following reagents were purchased: protected amino acids (Bachem, Inc., Torrance, CA, USA), [^{3}H]-Arginine, 30 ci per mmol (ICN), trifluoroacetic acid (TFA) (Sigma Chemical Co., St. Louis, MO, USA), trifluoromethane sulfonic acid (TFMS) (Aldrich Chemical Co., Metuchen, NJ, USA), Dowex 50 WX4 (Biorad Laboratories, Rockville Center, NY, USA), and scintillation fluid ACS (Amersham/Searle, Arlington Hts, IL, USA). Rabbit anti-tuftsin was generated by immunization with BSA-azo-phenylacetyl-Gly-Gly-tuftsin.[12] Phosphate-buffered saline (PBS) 0.15

M NaCl in 5×10^{-4} phosphate pH 7.4, sucrose solution 0.25 *M* sucrose in 5×10^{-4} *M* phosphate pH 7.4 were used as indicated.

Tuftsin (Thr-Lys-Pro-Arg) and its analogues were synthesized in our laboratory as previously described[20,21] utilizing the solid-phase method of Merrifield.[22] αNH_2 groups were Boc protected. ϵNH_2 groups of lysine and the guanido group of arginine were protected as the carbobenzoxy and tosyl derivatives, respectively. Activation of the carboxy group of the incoming residue was accomplished by dicyclohexylcarbodiimide (DCC). Deprotection of αNH_2 was done with 50% trifluoroacetic acid (TFA) in dichloromethane. All other groups including the peptide-resin ester bond were cleaved with one reagent, trifluoromethanesulfonic acid (TFMS) in anisole as described previously.[20,21] The released oligopeptides were purified on Dowex 50 columns using a pyridine-acetic acid linear gradient.[7] Labeled tuftsin, [^{3}H-Arg^4]-tuftsin, was synthesized according to Fridkin *et al.*[23] Essentially, Lys-Pro-O-Bzl, appropriately protected, was synthesized using DCC as coupling agent. N$^{\alpha}$-Boc-threonine was coupled to the deprotected N$^{\alpha}$ of the dipeptide. The proline-O-benzyl ester of the tripeptide was cleaved with sodium hydroxide and the peptide was coupled with DCC to N-hydroxysuccinimide to generate an active tripeptide ester, which was then coupled to [^{3}H]-arginine.[23] The tetrapeptide was deprotected with TFA and then with hydrogen with palladium on $BaSo_4$ as catalyst and purified on a Dowex column, followed by further purificaiton with high-performance liquid chromatography (HPLC) using 0.075% TFA in water as the mobile phase with unlabeled tuftsin as a marker. The stationary phase, a Zorbax column (duPont 0.94 × 25 cm) with trimethyl silane packing was used throughout.[5]

Binding studies to cell receptors were done on rabbit peritoneal neutrophils (PMN) harvested 24 hours after the intraperitoneal injection of 0.25% glycogen in 0.87% NaCl. PMN cells were washed 3 times with 5 volumes of PBS, counted, and appropriate aliquots incubated with [^{3}H-Arg^4]-tuftsin and competing analogues.

Antibodies were raised in rabbits by subcutaneous injections of BSA-azo-phenyl-acetyl-Gly-Gly-tuftsin in complete Freund's adjuvent. Binding to specific antibody was performed through the use of [^{3}H-Arg^4]-tuftsin and competing analogues to rabbit anti-tuftsin antiserum. This was then processed with the double antibody technique[12] (not shown) or fractionated with ammonium sulfate according to Farr[24] and the γ-globulin fraction counted as described in the respective legends.

RESULTS

Binding to Tuftsin Receptors and Tuftsin Antibody

The relative affinities of tuftsin and its analogues to both tuftsin receptors and tuftsin antibody were studied with labeled tuftsin [^{3}H-Arg^4]-tuftsin. These are represented graphically in FIGURE 2 and FIGURE 3. Competing oligopeptides were Thr-Lys-Pro, unlabeled tuftsin (Thr-Lys-Pro-Arg), [Pro-Pro^3]-tuftsin, (Thr-Lys-Pro-Pro-Arg), Ala-Lys-tuftsin (Ala-Lys-Thr-Lys-Pro-Arg), tuftsinyltuftsin (Thr-Lys-Pro-Arg-Thr-Lys-Pro-Arg), and Ala-Lys-tuftsin-Glu-Ala$_3$ (Ala-Lys-Thr-Lys-Pro-Arg-Glu-Ala-Ala-Ala). The following qualitative description applies equally to the binding characteristics of both the receptor for tuftsin and the antibody to tuftsin.

1. Two oligopeptide analogues had no measurable affinity and a third showed less affinity than tuftsin itself.

(a) The tripeptide lacking arginine did not bind to any significant extent. It has been known for some time that this tripeptide is not biologically active.[25] The inertness

of this analogue towards antibody binding was shown earlier in Fridkin's laboratory.[26] This finding was not unexpected since the peptide lacks arginine, the most determining residue in the immunizing hapten. It is also of prime importance to receptor binding.

(b) The other inert oligopeptide was the decapeptide Ala-Lys-tuftsin-Glu-Ala_3. Here, the tuftsin moiety is blocked at both termini by two or more residues. This analogue, again was not expected to bind either molecule since arginine is no longer the carboxyterminal residue.

(c) The hexapeptide, Ala-Lys-tuftsin, where the tetrapeptide is blocked at the amino terminus by the two naturally occurring residues in the sequence, Ala-Lys, but retains a free carboxy terminus, arginine, did exhibit good binding affinity but less than that of tuftsin.

2. Two other tuftsin analogues showed unexpectedly greater competitive affinity than tuftsin. Again, it should be stressed that the relative affinities of the two analogues were the same for receptor and antibody.

(a) The pentapeptide [Pro-Pro^3]-tuftsin, in which another proline residue is inserted penultimate to arginine, bound more strongly than tuftsin to both receptor and antibody. This pentapeptide was shown earlier to inhibit completely tuftsin stimulation of motility and phagocytosis of human blood granulocytes.[16,27] It was no surprise,

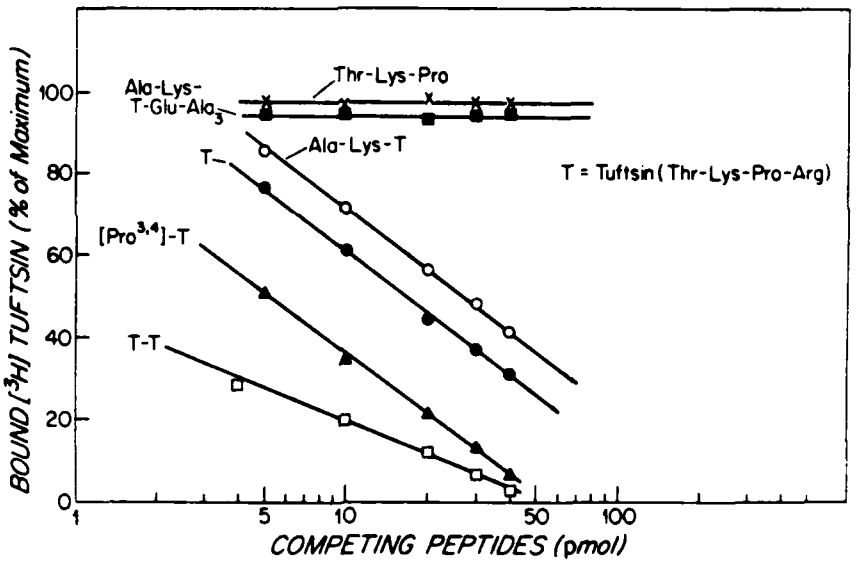

FIGURE 2. The relative affinities of tuftsin and its analogues to tuftsin receptors on rabbit peritoneal granulocytes (PMN).

PMN cells were washed with PBS followed by sucrose solution and suspended in the latter. All samples were run in triplicate. Each tube contained 3×10^6 cells, 10 p mol of [^{3}H-Arg^4]-tuftsin (200, 200 cpm) and increasing concentrations of competing analogues, final volume 200 μl; incubated for 30 min at 0° for maximum binding; centrifuged at 3000 × *g* for 10 min and the cells washed twice with 1 ml each of buffered sucrose. All manipulations so far were done at 0° 4°. To the washed cells, 300 μl of 1% SDS were added and heated to 90° for 90 min. Scintillation fluid, 3 ml, was added and the radioactivity counted. Nonspecific binding was determined by a chase with 10 nmol of unlabeled tuftsin. Specific binding (total counts less nonspecific counts) is shown in the figure as % of maximum binding (100%). Where only radioactive tuftsin (T) was used, the count yield was approximately 4500- 5500 cpm.

Thr-Lys-Pro (X—X); Ala-Lys-T-Glu-Ala_3 (■—■); Ala-Lys-T, (O- -O); T, (●—●); [$Pro^{3,4}$]-T (▲—▲); T-T (□—□).

The binding characteristics shown in this figure are representative of several experiments with fresh PMN cells obtained from several rabbits. For details see MATERIALS AND METHODS.

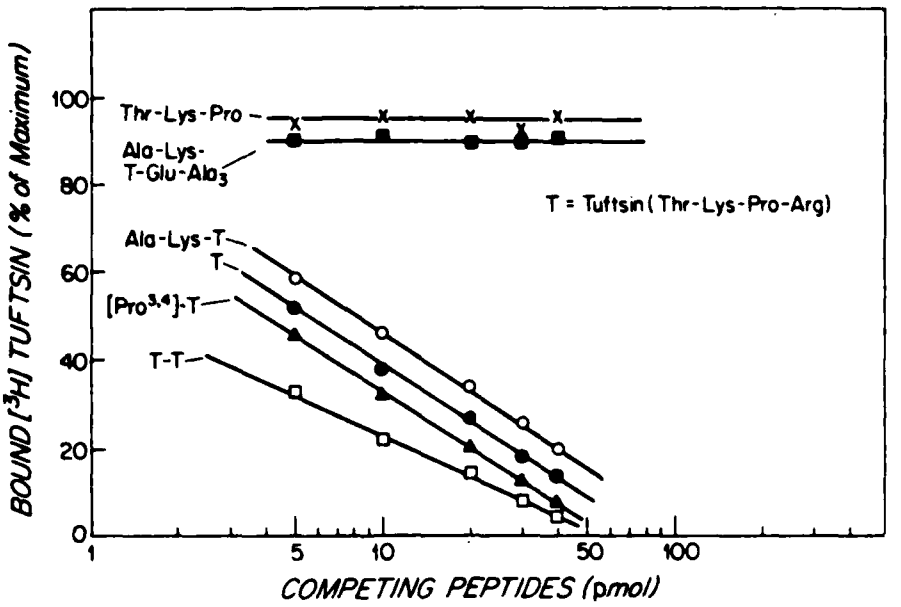

FIGURE 3. The relative affinities of tuftsin and its analogues to specific antibody to tuftsin.

Immunization of rabbits was done with 1 mg of antigen each in complete Freund's adjuvent at two-week intervals for six weeks. Serum was obtained 10 and 21 days after the last immunization.

Antiserum 0.4 ml (1:500) in PBS containing 0.05 *M* EDTA was incubated for 2 h at 0° with 5.0 p mol of [^{3}H-Arg^4]-tuftsin (100,100 cpm) and increasing concentrations of competing analogues also in PBS and EDTA, final volume 800 μl. An equal volume of saturated ammonium sulfate was added, centrifuged after 1 hr at 3,000 g for 10 minutes. The precipitate, washed twice in 400 μl each of saturated ammonium sulfate, was dissolved in 400 μl of water and 3 ml of scintillation fluid. Nonspecific binding was obtained by a 10-nmol chase. All samples were done in triplicate. The values are shown as % of maximum binding where only radioactive tuftsin (T) was used. One hundred percent specific binding gave approximately 3,000–3,500 cpm.

The oligopeptides tested were tuftsin Thr-Lys-Pro (X—X); Ala-Lys-T-Glu-Ala^3 (■—■); Ala-Lys-T (○—○); T, (●—●); [Pro^{3,4}-tuftsin (▲—▲); and T-T (□—□).

The binding characteristics shown in the figure are representative of several experiments with tuftsin antisera obtained from five different rabbits at different bleeding times. For details see MATERIALS AND METHODS.

therefore, to find that it binds receptors with a considerably greater affinity than tuftsin. However, its tighter binding to antibody was not expected because hapten analogues usually show less binding.

(b) Finally, the octapeptide tuftsinyltuftsin, showed the highest affinity of all the analogues tested for both tuftsin receptor and tuftsin antibody. FIGURE 2 and FIGURE 3 illustrate graphically the relative binding of the various oligopeptides discussed above. It is remarkable that the relative affinities obtained with tuftsin receptors are quite similar to those obtained with tuftsin antibody whether by the Farr technique or by double antibody technique using goat anti-rabbit γ-globulin.[12] This was repeatedly observed, a further indication that the two may be structurally related.

DISCUSSION

The specific antibody to tuftsin is shown in this study to display binding properties to tuftsin and its analogue receptors that are very similar to those shown by tuftsin

receptors in relative binding strength. The study was prompted by earlier observations that three analogues of tuftsin, Lys-Pro-Arg, Thr-Glu-Pro-Arg, and Thr-Lys-Pro-Pro-Arg, strongly inhibit the biological activity of tuftsin.[12,26,27] This inhibition is presumably due to stronger binding to tuftsin receptors on rabbit peritoneal granulocytes.[12] At the same time, these same analogues bound more strongly than tuftsin to rabbit antibody to tuftsin.[12,26,27] These results parallel those obtained by Becker and his colleagues.[28] They prepared antibodies in rabbits and rats against the chemotactic peptide, F-Met-Leu-Phe that was conjugated to a variety of carrier proteins. They then studied the characteristics of binding to the antibody by numerous analogues of the tripeptide. They found that there was a strong correlation between the strength of antibody binding to these analogues and their biological activity as judged by the induction of lysosomal enzyme. Our present data differ in only a few respects but strongly complement and support the data of Marasco *et al.*[28] In much the same manner, their elegant studies on the anti-idiotypic antibody[29] to the same tripeptide greatly amplify our early finding discussed in the introduction of this article.[3]

The antibody under study was directed against a natural activator of phagocytic cells[6,16] and binds only to these cells.[23,25,30] Furthermore, several of the analogues used are inhibitory to the biological activity of tuftsin and bind much more strongly to the receptor.

These studies, however, may be interpreted to indicate that the ability of the body to generate complementary binding sites to ligands is strictly limited. Thus, the combining site of an antibody generated against a ligand strongly mimics the receptor surface to the same ligand. This was shown quite early in our laboratory where an anti-idiotype against an antibody to the toxin lecithinase exhibited enzyme-like properties expressed in substrate binding characteristics.[3]

The similarities obtained in this study refer strictly to the binding characteristics of the oligopeptides and not to their biological activity. However, we showed earlier[5,12] that a correlation between antibody binding and biological activity does exist. [Glu^2]-tuftsin, [Pro-Pro^3]-tuftsin and tuftsinyltuftsin all inhibit phagocytic activity. They bind more strongly to antibody than tuftsin does. By contrast, the tripeptide analogue, Thr-Lys-Pro, does not bind and lacks biological activity.[5,7,12,14,25]

There appears to be an absolute requirement for the arginine residue since the tripeptide, Thr-Lys-Pro, is completely inactive. Blockage of both termini, as in Al-Lys-tuftsin-Glu-Ala_3, also renders the analogue inactive. However, blockage of the amino-terminal only, as in Ala-Lys-tuftsin, results in good binding but with lesser affinity than tuftsin. Variations in the third residue from arginine, as in Thr-Glu-Pro-Arg and Thr-Lys-Pro-Pro-Arg, considerably augments binding. The strongest binding was shown by tuftsinyltuftsin. This is not related to any change in residues but could be rationalized on the basis of a derivatized aminio-terminal. This would be akin to strong affinities shown to tuftsin antibody by acetyl-tuftsin, tyrosyl-tuftsin, *p*-aminophenylaacetyl-tuftsin, [Ala^1]-tuftsin, [Ser^1]-tuftsin, and [Lys^1]-tuftsin reported by Spirer *et al.*[26] These authors raised antiserum in rabbits with an identical procedure and further showed that the anti-allergic oligopeptide, Asp-Ser-Asp-Pro-Arg did not cross-react with tuftsin antiserum. They concluded that the dipeptide segment -Pro-Arg was not sufficient for good binding. However, the three-residue segment Lys-Pro-Arg was the necessary part for antigen recognition since substitution of Ser, Ala, Lys, for threonine or derivatization of threonine does not weaken the binding affinity. On the contrary, binding is augmented. In view of this, it is unlikely that a study of dipeptides and tripeptides with varying amino and carboxyterminals will yield additional and useful information.

One of the interesting pieces of information that this study revealed bears on the orientation of the tetrapeptide tuftsin towards its receptor site. The orientation towards the antibody site is surmised to be through the carboxy-terminal end. This results from

the attachment of the peptide to the carrier BSA through the amino-terminal end. Inasmuch as the binding characteristics of the receptor site parallel those of the antibody, it can be deduced that the orientation of tuftsin to its receptor site is also through the carboxy-terminal end. This may well be one of the few instances where the orientation of an active peptide towards its cell receptor can be deduced with a good measure of confidence.

SUMMARY

The characteristics of binding of tuftsin (Thr-Lys-Pro-Arg) and several of its synthetic analogues, to specific membrane receptors on rabbit polymorphonuclear granulocytes, were compared with the binding charcteristics of rabbit specific antibody to tuftsin. [^{3}H-Arg^4]-tuftsin was used as the principal ligand. Six analogues were studied. Two of these, Thr-Lys-Pro and Ala-Lys-tuftsin-Glu-Ala$_3$, showed no binding affinity either to receptor or antibody. Ala-Lys-tuftsin showed less binding than tuftsin to both acceptors. Three showed stronger binding than tuftsin. The order of binding among these was tuftsin ⟨[Glu]^2-tuftsin ⟨[Pro-Pro^3]-tuftsin ⟨tuftsinyltuftsin. This same order of binding was found with both receptor and antibody.

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On the Mechanism of the Augmentation of the Phagocytic Capability of Phagocytic Cells by Tuftsin, Substance P, Neurotensin, and Kentsin and the Interrelationship between Their Receptors[a]

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The discovery that the basic tetrapeptide tuftsin is the entity responsible for the phagocytosis-enhancing activity of leukophilic γ-globulins[1] and the recognition that tuftsin might have a physiological role in host defense mechanisms, led to extensive studies concerning potential tuftsin activities (for reviews see refs. 2–4). The cumulative efforts devoted to the understanding of the nature and extent of tuftsin activity will no doubt be duly reflected in the various chapters of this volume. With this in mind, we concentrate on work carried out by ourselves and our collaborators and do not attempt to give a comprehensive discussion of the various relevant aspects covered by work of others.

The synthesis of tuftsin and tuftsin analogues as well as of [^{3}H]-tuftsin opened up the possibility for structure-function analysis and identification and characterization of tuftsin binding sites.[3,5–7] In the following, we review our studies concerning: (1) the characterization of tuftsin binding sites on cells derived from the granulocyte-macrophage cell lineage;[8,9] (2) the mechanism by which tuftsin may modulate the functional capability of mouse macrophage and human polymorphonuclear leukocytes (HPMNL);[10] and (3) the binding and phagocytosis-enhancing activity of other natural peptides (substance P, neurotensin and kentsin) and some of their fragments and their interaction with the tuftsin receptors.[11–14]

TUFTSIN RECEPTORS ON MURINE MONONUCLEAR PHAGOCYTES

Binding studies using [^{3}H]-tuftsin have established that normal and stimulated peritoneal macrophages, as well as their precursor cells, peripheral blood monocytes and *in vitro* differentiated bone marrow-derived mononuclear phagocytes express specific binding sites, receptors, for tuftsin.[8,9] Binding characteristics were assessed in

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some detail for thioglycollate-elicited, cultured peritoneal macrophages. The binding of [^{3}H]-tuftsin at 22°C was rapid and saturable with an equilibrium dissociation constant (K_d) of 50–53 n*M*. The calculated number of binding sites for a thioglycollate-elicited macrophage was 72000. [^{3}H][N-Acetyl-Thr^1]-tuftsin, an inactive analogue of tuftsin, failed to bind specifically to thioglycollate-elicited macrophages. [N-Acetyl-Thr^1]-tuftsin and the tripeptide [Des-Arg^4]-tuftsin also failed to compete for tuftsin binding sites, while [D-Arg^4]-tuftsin, an analogue exhibiting a small tuftsin-like activity[5] exhibited a low degree of competition for specific tuftsin binding sites.[8] Normal as well as six different macrophage populations induced by i.p. injection of thioglycollate, concanavalin A, starch, mineral oil, glucan, and Bacilus Calmette Guerrin (BCG) exhibited a similar degree of [^{3}H]-tuftsin binding at two concentrations of the tetrapeptide (25 n*M* and 75 n*M*). Under similar experimental conditions, mouse fibroblasts and lymphocytes revealed no detectable specific binding. A macrophage-like tumor cell line, P388D1 was also shown to express specific binding sites for [^{3}H]-tuftsin.[9] Tuftsin binding sites on thioglycollate-elicited macrophages are trypsin sensitive. A 15-min treatment with 0.4 mg/ml of trypsin at 37°C reduced the specific binding of tuftsin to about 12% of control values. The nonspecific component of the binding was not affected by trypsin treatment. A 6-hr culture period in culture medium containing 10% heat-inactivated fetal calf serum sufficed for restoration of 58% of the specific binding (Bar-Shavit and Goldman, unpublished observations).

SUBSTANCE P, NEUROTENSIN, AND KENTSIN AS COMPETITORS OF TUFTSIN FOR ITS BINDING SITES

Although binding competition studied with tuftsin analogue and derivatives have suggested the existence of a rather high structural specificity at the specific tuftsin binding sites, studies with several natural peptides and some of their peptide fragments suggested a need for expansion of the formulated requirements for specific binding.

Substance P (SP) and its NH_2-terminal tetrapeptide fragment [SP (1–4)] displace

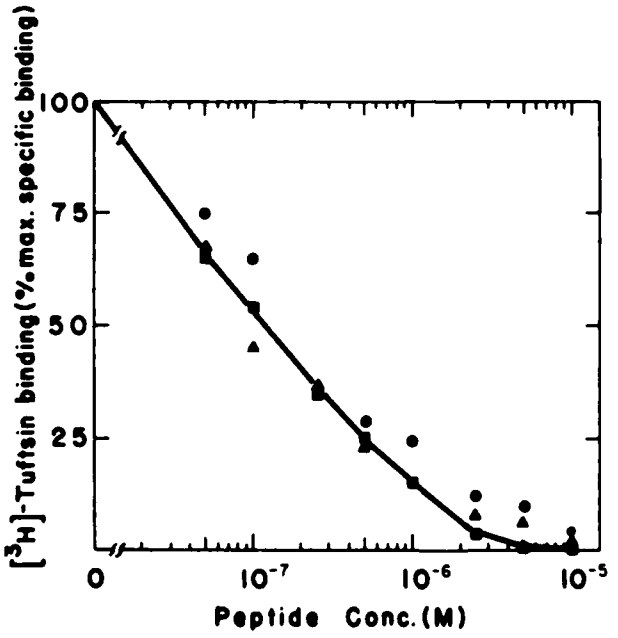

FIGURE 1. Effect of SP, (●), and SP(1–4), (▲), on the binding of [^{3}H]-tuftsin to thioglycollate-elicited macrophages. The solid line represents the competition exhibited by unlabeled tuftsin, (■). From Bar-Shavit *et al.*[11] Reproduced with permission of Academic Press, Inc.

TABLE 1. Competition of Kentsin and Neurotensin for Specific Tuftsin Binding Sites[a]

Unlabeled Peptide (Conc. μM)		[^{3}H]-tuftsin Bound/Well cpm	Specific [^{3}H]-tuftsin Bound/Well cpm	% Inhibition of Specific [^{3}H]-tuftsin Binding
—		2204 ± 102	1522	
Tuftsin[b]	0.5	874 ± 32	192	87
	5.0[c]	682 ± 71	0	100
Kentsin	0.5	1036 ± 114	354	77
	5.0	816 ± 88	134	91
Neurotensin	0.1	1641 ± 114	959	37
	0.5	1017 ± 77	335	78
	5.0	819 ± 32	137	91
N-formyl-Methionyl--Leucyl-Phenylalanine	5.0	2141 ± 197	1459	4

[a]Binding competition studies were carried out essentially as described.[8] Thioglycollate-elicited macrophages (7×10^5/well) were cultured for 48 hr in a 24-well tissue culture plate (Costar). The specified unlabeled peptides and [^{3}H]-tuftsin (specific activity 24 Ci/mmol) (a gift from Drs. P. Gottlieb and M. Fridkin) at a 50 nM concentration were added consecutively in a final volume of 0.5 ml Dulbecco's phosphate-buffered saline. The binding assay was carried out for 35 min at 22°C.

[b]Tuftsin was a gift from Drs. P. Gotlieb and M. Fridkin. Kentsin and the formyl peptide were purchased from Sigma Chem. Corp. (St. Louis, MO) and neurotensin was purchased from Vega Biochemicals (Tucson, AZ).

[c]The residual [^{3}H]-tuftsin binding at a tuftsin concentration of 5 μM was defined as the nonspecific component of the binding, and was substracted from all binding data.

[^{3}H]-tuftsin from its specific binding sites on thioglycollate-elicited macrophages with the same inhibition constant (K_i) as unlabeled tuftsin (FIG. 1).[11] [^{3}H]-tuftsin was also displaced from its specific binding sites by kentsin and neurotensin (NT) but not by the chemotaxis-inducing peptide N-formyl-methionyl-leucyl-phenylalanine (TABLE 1). The interrelationship between tuftsin binding sites and specific binding sites for substance P, neurotensin, and several of their peptide fragments is also strongly suggested from binding competition studies using [^{3}H]-neurotensin ([^{3}H]-NT). A Scatchard analysis of the binding curve for [^{3}H]-NT on thioglycollate-elicited macrophages revealed two subclasses of binding sites, a minor population of high-affinity binding sites (K_d 0.9 nM and 4800 sites/cell) and a major population of lower binding affinity (K_d 28 nM and 33500 sites/cell).[12,13]

Tuftsin, SP, SP(1–4), the COOH-terminal hexapeptide fragment of NT [NT (8–13)], and the COOH-terminal octapeptide fragment of NT [NT(6–13)] were shown to compete with [^{3}H]-NT for its binding sites, whereas the NH_2-terminal decapeptide fragment of NT [NT(1–10)], the COOH-terminal heptapeptide fragment of SP [SP(5–11)], and the COOH-terminal tripeptide fragment of NT [NT (11–13)] were rather ineffective (FIG. 2). The sequences of the above peptides and fragments are given in TABLE 2.

The K_i values that were calculated for the inhibition of [^{3}H]-NT binding by the various effective competitive peptides and by unlabeled NT were all in the same concentration range.[12] The K_i for NT, NT(8–13), NT(6–13), SP, SP(1–4) and tuftsin were 12, 13, 13, 20, 17 and 16 nM, respectively.[12,13] It is of note that although the calculated K_i values for all the above peptides were remarkably similar to that obtained for NT, only NT was efficient in inhibiting 100% of the binding of [^{3}H]-NT. We suggest that the residual binding of [^{3}H]-NT of 10–20% that cannot be displaced by

NT fragment peptides, SP, and tuftsin reflects the component of the high-affinity binding sites, and that the displaceable component reflects the major site population of lower affinity, which appears to be shared by the various peptides described. *In vitro* differentiated bone marrow-derived macrophages also express two subclasses of affinity sites for [^{3}H]-NT[13] and tuftsin is capable of displacing 90% of the specific binding (Bar-Shavit and Goldman, unpublished observations).

AUGMENTATION OF THE PHAGOCYTIC CAPABILITY OF MACROPHAGES AND HPMNL

Constantopoulos and Najjar[15] have shown that tuftsin stimulates the phagocytic activity of guinea pig peritoneal granulocytes, rabbit lung, and mouse peritoneal

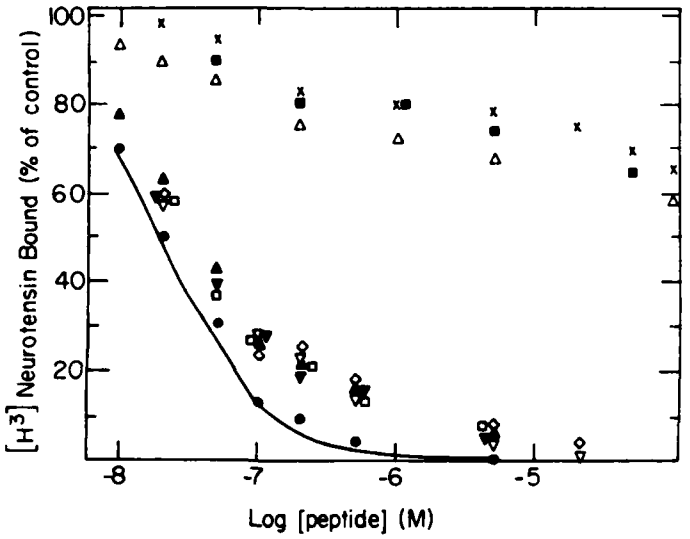

FIGURE 2. Competitive inhibition of [^{3}H)-neurotensin ([^{3}H]-NT) bindirg by unlabeled neurotensin (NT), (●); its partial sequences NT(8–13), (▽); NT(6–13), (◇); NT(11–13), (x); NT(1–10), (Δ), substance P (SP), (▲); SP(1–4), (□); SP(5–11), (■); and by tuftsin, (▼). Binding is expressed as percent of specific [^{3}H]-NT binding in the absence of unlabeled peptides. [^{3}H]-NT was at a constant concentration of 20 n*M*. From Bar-Shavit *et al.*[12] Reproduced with permission of Churchill Livingstone.

macrophages, and HPMNL to a similar extent. Maximum stimulation was observed at about 0.1 μg tuftsin/ml (160 n*M*) and amounted to an increase in the percent of cells phagocytizing opsonized *Staphylococcus aureus* from an average of 12–16 to an average of 27 to 33 for the different phagocytic populations studied.[15] Since the assay was carried out under rather limited conditions (2 bacteria per phagocyte), no information regarding the increased phagocytic capability of phagocytizing cells could be gained.

Normal and thioglycollate-elicited macrophages ingest heat-killed (HK) yeast cells and IgG-coated sheep red blood cells (IgGSRBC). Our studies demonstrate that the ingestion of both particles, which involves different recognition sites on the plasma membrane, is augmented up to two fold by tuftsin (optimal effect obtained already at about 0.1 μg/ml). Moreover the overall augmented phagocytic capability is a

TABLE 2. The Sequence of Tuftsin, Kentsin, Substance P (SP), Neurotensin (NT), and Some of Their Fragments[a]—Peptides Sharing the Capacity to Augment the Phagocytic Capability of Phagocytic Cells

Tuftsin[1,b]	Thr-Lys-Pro-Arg
Kentsin[45]	Thr-Pro-Arg-Lys
SP(1–4)	Arg-Pro-Lys-Pro
SP[40]	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met(NH_2)
NT(8–13)	Arg-Arg-Pro-Tyr-Ile-Leu
NT(6–13)	Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu
NT(1–10)	pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro
NT[40]	pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu

[a]All amino acids are in the L-configuration.
[b]Superscripts denote references for the sequence.

reflection of both an increase in the number of macrophages that phagocytize particles and an increase in the average number of particles ingested by phagocytizing cells.[8] For example, the average number of IgGSRBC ingested by thioglycollate-elicited macrophages in the presence and absence of tuftsin (1 μg/ml) was 380 and 150, respectively, and the percentage of ingesting cells increased from 38 to 65. In resident peritoneal macrophages, the effect was even more pronounced. The percentage of cells phagocytizing IgGSRBC increased from 85 to 90 whereas the average number of ingested IgGSRBC increased from about 300 to 480 in the presence of tuftsin. A dose-response curve for the augmentation of phagocytosis of [^{3}H]-zymosan particles is shown in FIGURE 3. The half-maximal augmenting effect of the phagocytic capability was obtained at about 22 n*M* of tuftsin,[12] a concentration at which a rather high degree of occupancy of the tuftsin binding sites was obtained. The optimal augmenting effect was obtained at a tuftsin concentration of 50–100 n*M* at which tuftsin binding reaches 50–100% of the saturation value. Thus for optimal expression of the activity of tuftsin, there is a requirement for a high degree of receptor occupancy. An essentially similar pattern of augmentation of the phagocytic capability of thioglycollate-elicited macrophages was observed with the peptides that were shown to compete with tuftsin for its binding sites.[12] The concentrations at which half-maximal phagocytosis-augmenting activity of SP, NT(8–13), and NT(6–13) was observed were 26 n*M*, 18 n*M*, and 18 n*M*, respectively (FIG. 3). A similar dose-response curve was obtained for the SP-augmented phagocytic capability of resident macrophages (using HK yeast cells as particles).[11] Kentsin is also effective in augmenting the phagocytic response of thioglycollate-elicited macrophages. The dose-response curve of the augmenting effect

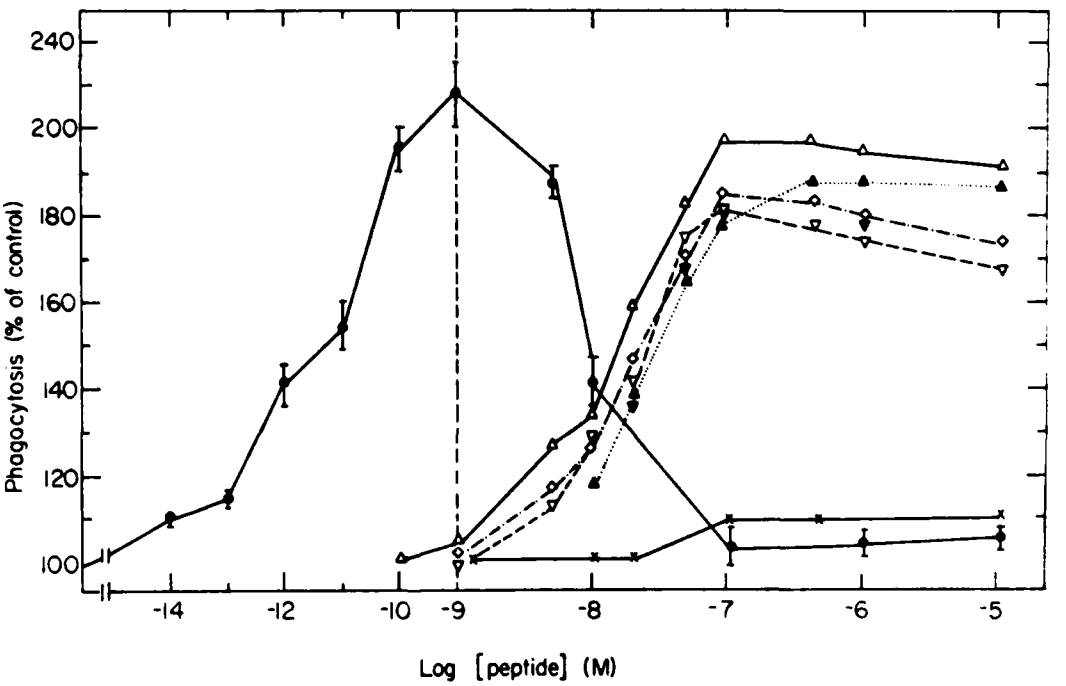

FIGURE 3. Dose-response curves for the effect of tuftsin, (▼); neurotensin (NT), (●); NT(1–10), (Δ); NT(8–13), (▽); NT(6–13), (◊); NT(11–13), (x); and substance P (SP), (▲) on the phagocytic capability of thioglycollate-elicited macrophages. The data represent the phagocytosis of [^{3}H]-zymosan particles. From Bar-Shavit *et al.*[12] Reproduced with permission of Churchill Livingstone.

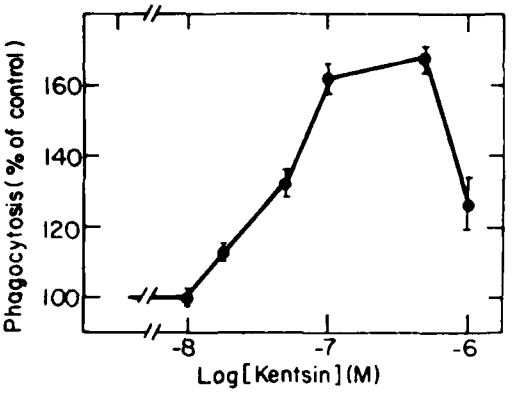

FIGURE 4. The effect of kentsin on the phagocytosis of [^{3}H]-zymosan particles by thioglycollate-elicited macrophages. Cell cultivation and the assays of phagocytosis were carried out as described.[12] The graph summarizes results of 4 experiments with each point carried out in quadruplicate ±SEM. The average value of 100% phagocytosis in the 4 experiments was 37668 ± 2250 dpm per 2×10^5 macrophages.

(FIG. 4) is similar to that observed for tuftsin, SP, and the other active peptides described. At 1 μM, the augmenting activity of kentsin decreased to a certain extent. Of all the peptides that were shown above to displace [^{3}H]-tuftsin from its binding sites, neurotensin is unique in its effect on the phagocytic capability of thioglycollate-elicited macrophages[12] and HPMNL.[14] The biphasic dose-response curve of the augmentation of phagocytosis shown in FIGURE 3, suggests that the optimal augmentation of the phagocytic capability occurs at about 1 nM of NT. The half-maximal augmenting effect is brought about by a NT concentration of 0.01 nM, a concentration at which there is a minimal occupancy of the high-affinity NT binding sites (K_d 0.9 nM). The descending limb of the biphasic dose-response curve reaches a value of 50% decline in the augmented response at about 10 nM (FIG. 3). A return to the basal phagocytic capability of thioglycollate-elicited macrophages is reached at 100 nM, a concentration at which tuftsin exhibits its maximal effect. At 10 nM of NT, the occupancy of the relatively low-affinity sites (K_d 28 nM) is already significant. The occupancy of the low-affinity sites appears to lead to inhibition of the augmentation of phagocytosis brought about by occupancy of the high-affinity sites. The occupancy of the relatively low-affinity sites by NT (at 100 nM) is, however, inhibitory also to the activity of peptides that augment the phagocytic response by interaction with tuftsin binding sites; for example, the phagocytosis-augmenting activity of SP (at 100 nM) is totally abrogated by 100 nM of NT.[12]

The data of both the binding competition studies and the phagocytosis assays suggest that the relatively low-affinity NT binding sites are identical with the tuftsin binding sites, and that NT acts as an inhibitory tuftsin analogue at these sites. A variety of tuftsin analogues have been shown to be inhibitors of tuftsin activity.[7] Most notable is the pentapeptide, Thr-Lys-Pro-Pro-Arg,[3,4] that frequently contaminates tuftsin preparations, the presence of which even as a minor contaminant abolishes the effects of tuftsin.

Our studies indicate that, similar to macrophages, the phagocytic capability of HPMNL is augmented by SP and SP(1–4). The ingestion of both HK yeast cells and IgGSRBC is augmented in a dose-dependent manner and the half-maximal effect is attained at a higher concentration than needed for a similar effect in macrophages,[11] in line with a lower affinity of tuftsin receptors on HPMNL.[5] The phagocytic response of HPMNL is also augmented by NT. A rather complex dose-response relationship for the augmentation of the phagocytic capability was observed.[14] A biphasic dose-response curve with an optimum in the range of 0.1–1 nM and a second concentration range in which the phagocytic capability is augmented with an optimum about 10–100 nM depending on the donor of the cells was seen.

ON THE MECHANISM OF ACTION OF TUFTSIN, SP, AND NT

Intracellular levels of cyclic nucleotides have been shown to influence the phagocytic response of mammalian leukocytes. cAMP and pharmacological agents that elevate cAMP levels were shown to inhibit phagocytosis whereas cGMP and cholinergic agonists were shown to stimulate phagocytosis.[10,16,17] We have, therefore, explored the possibility that tuftsin binding may affect the intracellular levels of cGMP and cAMP. FIGURE 5 shows that indeed, tuftsin binding to both thioglycollate-elicited macrophages and HPMNL leads to dose-dependent changes in the intracellular levels of both cyclic nucleotides. Intracellular cGMP concentration increases by about 80–90% whereas that of cAMP decreases by about 20–25% in both cell types. The half-maximal response of HPMNL and thioglycollate-elicited macrophages is obtained at tuftsin concentrations of 80–90 n*M* and 30–40 n*M*, respectively, and is in accord with the concentrations required for half-maximal augmentation of the

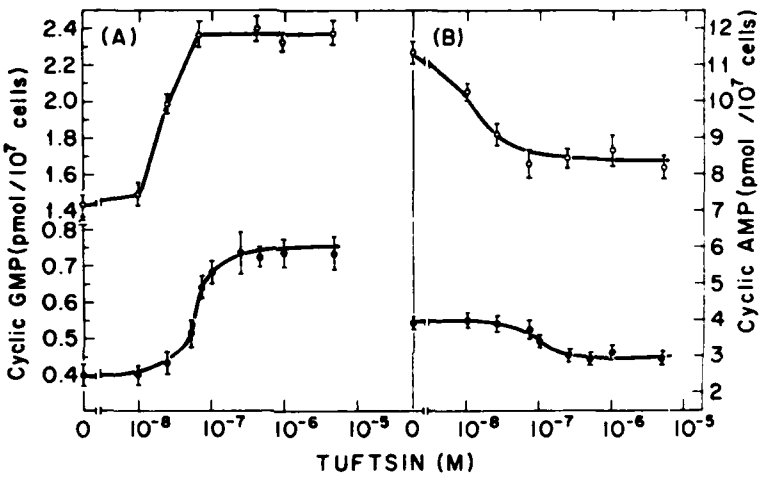

FIGURE 5. The dependence of intracellular levels of cGMP(A) and cAMP(B) in HPMNL (●) and thioglycollate-elicited macrophages (O) on tuftsin concentration. Tuftsin at the specified concentration was incubated with cells for 20 min at 37°C and cyclic nucleotides were assayed. Each point represents the mean of three separate incubation experiments. From Stabinsky *et al.*[1] with permission of the American Society for Microbiology.

phagocytic response. The maximal effect in both cell types does not decline with increasing concentrations of tuftsin up to 5 μM. The changes in the levels of the cyclic nucleotides are detectable already 4 min after the addition of tuftsin, and persists in the presence of the peptide for at least 60 min (FIG. 6).

Increase in the intracellular level of cGMP is generally attributed to indirect effects of hormones, neurotransmitters, and membrane-active agents on the activity of cellular guanylate cyclase.[18] Schultz *et al.*[19] have suggested that an increase in intracellular cGMP may reflect changes in the intracellular concentration or distribution of Ca^{2+}, and indeed in several systems a Ca^{2+}-mediated activation of guanylate cyclase and cGMP accumulation were observed.[20,21] Further support for the notion that increased intracellular cGMP levels correlated with elevation of intracellular Ca^{2+} comes from experiments showing that interaction with the ionophore A23187 leads to augmentation of intracellular cGMP.[18]

In view of the above, we explored the possibility that tuftsin causes changes in the

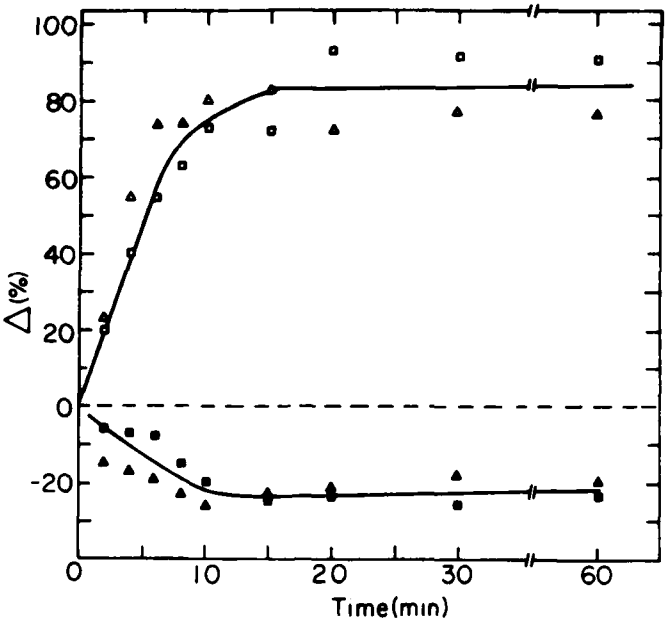

FIGURE 6. Time course of tuftsin-induced modulation of intracellular cyclic nucleotides. Thioglycollate-stimulated mouse macrophages (□, ■) and HPMNL (Δ, ▲) were incubated in the presence or absence of 250 n*M* of tuftsin at 37°C for the indicated time intervals. cGMP (Δ, □) and cAMP (▲, ■) were determined as described.[10] Zero time values in pmol/10^7 phagocytes of control cell suspensions amounted to: cGMP in macrophages 1.4, and in HPMNL 0.38; cAMP in macrophages 8.9 and in HPMNL 4.3. Adapted from Stabinsky *et al.*[10]

intracellular concentration of Ca^{2+} by changing Ca^{2+} fluxes across the cell membrane. Using $^{45}Ca^{2+}$, we were not able to observe any change in the rate of influx of $^{45}Ca^{2+}$ in the presence and absence of tuftsin. The rate of release of exchangeable $^{45}Ca^{2+}$ from $^{45}Ca^{2+}$ preloaded cells was, however, significantly increased in thioglycollate-elicited macrophages in response to interaction with tuftsin (FIG. 7). The effect on the release of $^{45}Ca^{2+}$ was specific for tuftsin, and was not observed with inactive analogues of tuftsin.[10] An increased efflux of $^{45}Ca^{2+}$ from preloaded cells could reflect a transient increase in free intracellular calcium ions caused by a shift of Ca^{2+} from membrane

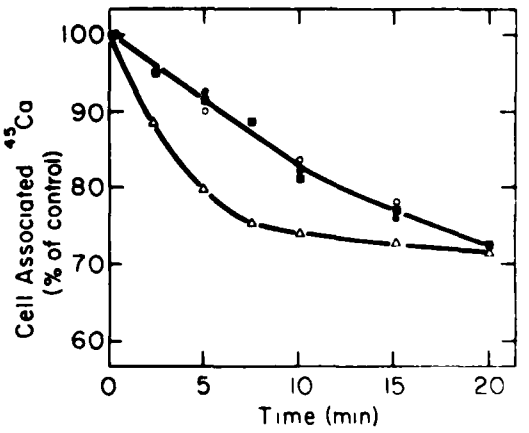

FIGURE 7. The effect of tuftsin and tuftsin analogues on the efflux of $^{45}Ca^{2+}$ from thioglycollate-elicited macrophages. Macrophage monolayers preloaded with $^{45}Ca^{2+}$ were incubated in the absence, (■) or presence of the tested peptide at 250 n*M* for the indicated time intervals. (Δ), tuftsin; (○), [N-acetyl-Thr^1]-tuftsin; (●), [Des-Thr^1]-tuftsin. From Stabinsky *et al.*[10] With permission of the American Society for Microbiology.

sites into the cytosol. This shift also activates, among a variety of Ca^{2+}-dependent proteins, the plasmalemmal Ca^{2+} extrusion system.[22,23]

Direct measurements of Ca^{2+} fluxes were performed by measuring changes in extracellular Ca^{2+} concentrations with the help of a very sensitive Ca^{2+}-selective electrode/amplifier/recorder system as previously described.[22-24] In those studies, we were able to show that SP(1–4) brings about a dose-dependent release of cellular Ca^{2+} (increased efflux of Ca^{2+}) (FIG. 8) from HPMNL (D. Romeo and R. Goldman, unpublished observations). The rate of release increased from 1–100 n*M* of SP(1–4). At 500 n*M*, HPMNL were refractory to the Ca^{2+}-releasing effect of SP(1–4). NT was also shown to be a potent inducer of Ca^{2+} efflux from HPMNL.[14] The dose-response curve of the release was found to be biphasic with an optimum at 0.1 n*M* NT in the incubation medium. NT concentrations above 0.1 n*M* led to a decreased Ca^{2+} efflux that reached basal levels (those of control cells) at about 1–10 n*M* of NT. The kinetics of Ca^{2+} efflux are shown in FIGURE 8. The SP(1–4)- and NT-induced extrusion of Ca^{2+} from HPMNL proceeded rather slowly, obtaining a steady state after several minutes. Ca^{2+} release elicited by the ionophore A23187 (FIG. 8) and a phorbol diester (PMA)[22] was rapid and did not require an extended lag period. The dose-response

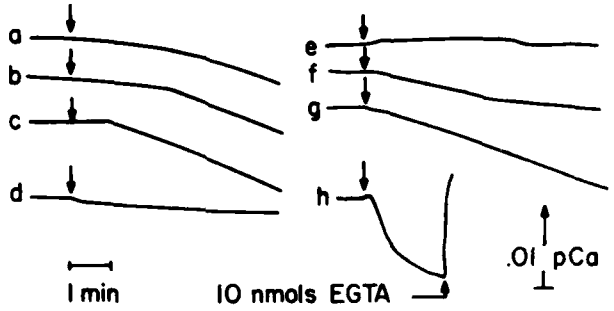

FIGURE 8. Kinetics of Ca^{2+} extrusion from HPMNL exposed to the NH_2-terminal tetrapeptides fragment of substance P [SP(1–4)] and neurotensin (NT). a, b, c and d correspond to 1 n*M*, 10 n*M*, 100 n*M*, and 500 n*M* of SP (1–4), respectively. e corresponds to controls in which buffer was added, and f and g correspond to 3.3 n*M* and 0.66 n*M* of NT, respectively. h corresponds to addition of 2 μM of A 23187. Experimental details are exactly as described.[14] The data concerning the effects of SP(1–4) are from D. Romeo and R. Goldman, unpublished observations. The kinetics of extrusion induced by NT is taken from Goldman *et al.*[14]

curves of Ca^{2+} efflux induced by both SP(1–4) and NT are in accord with the dose-response curves for the augmentation of the phagocytic capability of HPMNL. At concentrations in which the peptides cause optimal Ca^{2+} release, both also have an optimal phagocytosis-augmenting effect. Of special interest is the observation that the concentration range that marks decline in the augmentation of the phagocytic capability coincides with a decline in the Ca^{2+}-releasing effect.

The increased rate of exchange of $^{45}Ca^{2+}$ induced in thioglycollate-elicited macrophages by tuftsin and the net efflux of Ca^{2+} from HPMNL induced by SP(1–4) and NT are likely to be rather early responses of the cells to peptide-receptor interaction. Assuming that they reflect the activity of the Ca^{2+} pump[22,23] embedded in the plasma membrane, the fluxes give an indirect measure of a shift of intracellular Ca^{2+} from membrane sites into the cytosol. Release of Ca^{2+} from membrane sites as a result of interaction of HPMNL with phagocytizable particles and chemotactic peptides was demonstrated directly in ultrastructural studies using electron microscopy.[25]

CONCLUDING REMARKS

Ca^{2+} and Cyclic Nucleotides as Mediators of Tuftsin's Induced Cellular Activities

Increased levels of intracellular Ca^{2+} seem to correlate rather well with increased levels of cGMP and decreased levels of cAMP in a variety of cell systems.[26] The interrelationship of these intracellular changes with a variety of macrophage and HPMNL functions have been studied in some detail. For example, exogeneous agents that increase cAMP inhibit locomotion,[27,28] chemotactic responsiveness,[27,28] nitroblue tetrazolium reduction,[29] and granule discharge,[30,31] whereas agents that increase intracellular cGMP stimulate those processes.[28-33] Chemoattractants, on the other hand, increase the intracellular level of free Ca^{2+}.[25,34,35] Intracellular cyclic nucleotide levels have also been shown to be critical in the phase of macrophage activation for cytotoxicity towards tumor cells.[36]

On the basis of the above, it is rather tempting to suggest that tuftsin and other biologically active peptides that interact with cells via the tuftsin receptors, or via different receptors but inducing similar changes in intracellular cyclic nucleotides levels, would also affect locomotion, granule discharge, and activation processes in phagocytes.

In support of the above are the findings that tuftsin increases the bactericidal activity of neutrophils and macrophages and the tumoricidal activity of the latter.[3,4] Tuftsin has also been shown to increase nitroblue tetrazolium reduction in HPMNL[37] and to stimulate the migration and be a chemotaxin for human mononuclear cells and neutrophils. The latter observations are, however, rather controversial (for reviews see refs. 3, 4). SP has been shown to be a chemoattractant and to cause granule discharge in HPMNL[38] and NT to be a chemokinesis inducer and possibly also a chemotaxin for HPMNL.[14]

Structure-Function Relationships at Tuftsin Binding Sites

Numerous studies have been carried out with the purpose of defining the structural requirements for binding and activity of tuftsin analogues. The stimulation of phagocytosis is generally found to be diminished by any alteration, substitution, or addition to the basic structure,[3,5,6,8] but some conflicting results were also reported.[3] Of note is also the finding that partial sequences of histones, tetrapeptides that do not carry any resemblance to tuftsin, also exhibit tuftsin-like activity.[39] Most of these studies are limited to assays of stimulation of phagocytic capability and do not prove the identity of the site of interaction with tuftsin binding sites. In our studies, we have shown that kentsin, SP, NT, and some of their peptide fragments (TABLE 2) share both the activity of tuftsin as augmentors of the phagocytic capability of macrophages and HPMNL and the same binding sites (inferred from binding competition studies). The active peptides appear to have very little in common except for two basic amino acids and a proline residue in a variable sequence and a nonconserved NH_2 or COOH terminus. The only peptide (from TABLE 2) that interacts with the tuftsin binding sites on macrophages and appears to inhibit the stimulatory activity associated with occupancy of these sites is NT. NT could be a competitive inhibitor of the stimulation via the tuftsin receptor sites. But on the other hand, this phenomenon could reflect a desensitization effect, caused by the prior saturation of the high-affinity NT binding sites, which leads to a refractory response upon occupation of tuftsin binding sites.

It is worth noting that SP and NT are widely distributed in both the central nervous system and peripheral tissues where they exhibit a broad spectrum of pharmacological

effects.[40-43] A whole range of pharmacological activities of SP depends on the sequence of the COOH-terminal six or seven amino acids,[44] whereas those of NT require an intact COOH-terminal tripeptide.[42,43] The structural element that is involved in the augmentation of the phagocytic capability of phagocytic cells by SP resides in the NH_2-terminal tetrapeptide, a portion of the molecule that seems to be irrelevant to other biological activities induced by SP. In the apparently more complex interaction of NT and its fragment peptides with macrophages, our studies suggest that an intact NH_2-terminal end is required for interaction with the high-affinity NT binding sites. Of the two peptides that appear to interact with these sites, NT and NT(1–10), the former interacts with a much higher affinity. Elements in the COOH-terminal end are required for the interaction with the relatively low affinity sites that are probably identical with tuftsin binding sites.

The physiological relevance of the interaction of SP and NT with macrophages and HPMNL is not entirely clear. Both neuropeptides/neurohormones were shown to interact with mast cells[46,47] and to be strong vasodilators.[40,43] Both affect the mobilization of HPMNL[14,38] and enhance the phagocytic capability of macrophages and HPMNL.[11,12,14] SP is released from sensory nerves following injury, axon reflex, and antidromic stimulation and thus is thought to be involved in neurogenic inflammation.[46,48] The physiological levels of NT in the blood are in the range that we have shown effective *in vitro* in the augmentation of the phagocytic capability of macrophages and HPMNL, and is further increased after fat intake.[49] Thus, SP and NT have been shown to have properties and be located or released at sites that will enable them to be instrumental as mediators at various stages of inflammatory reactions.

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Central Effects of Tuftsin

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INTRODUCTION

At the beginning of our collaboration with Dr. Siemion's group on the analgesic effects of the new enkephalin analogues,[1] Dr. Siemion supplied us with a sample of tuftsin synthesized in his laboratory. He suggested examination of tuftsin's effects on the central nervous system. Since this tetrapeptide, threonyl-lysyl-prolyl-arginine, discovered in the laboratory of Dr. Najjar[2-4] was completely different in structure from enkephalins, tyrosyl-glycyl-glycyl-phenyloalanyl-leucine or methionine, the first experiments were performed with skepticism. Tuftsin was injected intracerebroventricularly into rats, and the reaction of animals to nociceptive stimulus was measured by means of the hot-plate method. To our surprise, in several repeated experiments it was observed that tuftsin induced a short-term antinociceptive effect. The aim of this paper is to report the analgesic action of tuftsin and its analogues, the effort to elucidate the mechanism of this effect, and the influence of tuftsin on the behavior and cardiovascular system after intracerebroventricular administration into rats.

MATERIALS

Experiments were carried out on male Wistar rats (200–220 g) and on male Porton mice (10–20 g) from the Central Animal Farm of the Silesian School of Medicine.

The following substances were used: tuftsin and tuftsin analogues synthesized in the Institute of Chemistry of Wrocław University. Synthetic bradykinin (BRS 640) was kindly donated by Sandoz (Basle), and naloxone hydrochloride (NAL) by Endo Laboratories, Garden City, NY.

METHODS

Means of Injection

Tuftsin and tuftsin analogues were dissolved in physiological saline solution (SAL), BRS was used as original solution from ampules. All these substances were administered without narcosis into the right lateral ventricle of the brain of rats in a

volume of 10 μl or in mice,[6] in a volume of 1–3 μl. BRS was always injected 10 min before tuftsin administration. Control animals were injected by SAL i.c.v. in the same volume as experimental animals. All solutions injected i.c.v. had a pH of 7.4. NAL dissolved in SAL was administered intraperitoneally (i.p.) in a dose of 2.5 mg/kg in a volume of 1 ml/kg, 30 min before injection of tuftsin or its analogues. Control animals received the solvent in the same volume i.p.

Antinociceptive Effect

Rats or mice were injected i.c.v. with tuftsin or tuftsin analogues in a dose of 200 μg or BRS in a dose of 1–3 μg before tuftsin or SAL. Immediately after tuftsin, tuftsin analogue, or SAL injection, animals were placed on the hot plate.[7] A licking of the front, and hind paws was used as the endpoint for the determination of response latencies recorded to the nearest 0.1 sec. If a latency time was longer than 30 sec, the animal was removed from the hot plate to avoid heat burn and the latency time assumed to be 30 sec. The other groups of rats were pretreated by NAL before tuftsin or tuftsin analogue administration. The latency time of reaction on the nociceptive stimulus was measured every 5–10 min during the first hour and then every 20–30 min until 210 min after injection of tuftsin or tuftsin analogues.

Behavior

Immediately after tuftsin or SAL administration, each rat was placed into a circular cage 80 cm in diameter and 30 cm in height. The floor and walls of the cage were stainless steel. Three cm above the floor, photoresistors and light beams were fixed. On the floor, holes 3 cm in diameter were located at equal distances. A photoresistor was placed under each hole. During horizontal movements of rats and when rats dipped their heads into the hole, impulses from photoresistors were registered by separate counters. When a rat put its forepaws on the wall of the cage (rearing) the circuit of the very low current from the floor and the wall was closed and amplified, and the impulse was registered by the counter. Behavioral episodes were automatically recorded every 5 min during the first 15 min, then every 15 min up to 1 hr, and every half hour to the end of the 2 hr observation period. The episodes of dipping the head into the hole and of rearing were assessed as exploratory activities, and horizontal movements as general locomotor activity.

Cardiovascular System

In the rats under urethane anesthesia (1.5 g/kg i.p.), the blood pressure from the right carotid artery was registered by means of a mercury manometer on the kymograph. The number of heartbeats was measured electrocardiographically. The number of respirations/min was counted. After 10 min of adaptation, the control measurements were recorded. Then tuftsin was administered i.c.v. in a dose of 200 or 400 μg and the effects were observed for 25 min. In separate groups of animals, BRS (3 μg i.c.v.) was administered 15 min before injection of tuftsin, or NAL (1.5 mg/kg i.p.) was injected 30 min before tuftsin administration and the same parameters were recorded. Results of all experiments were statistically analyzed using Student's t test.

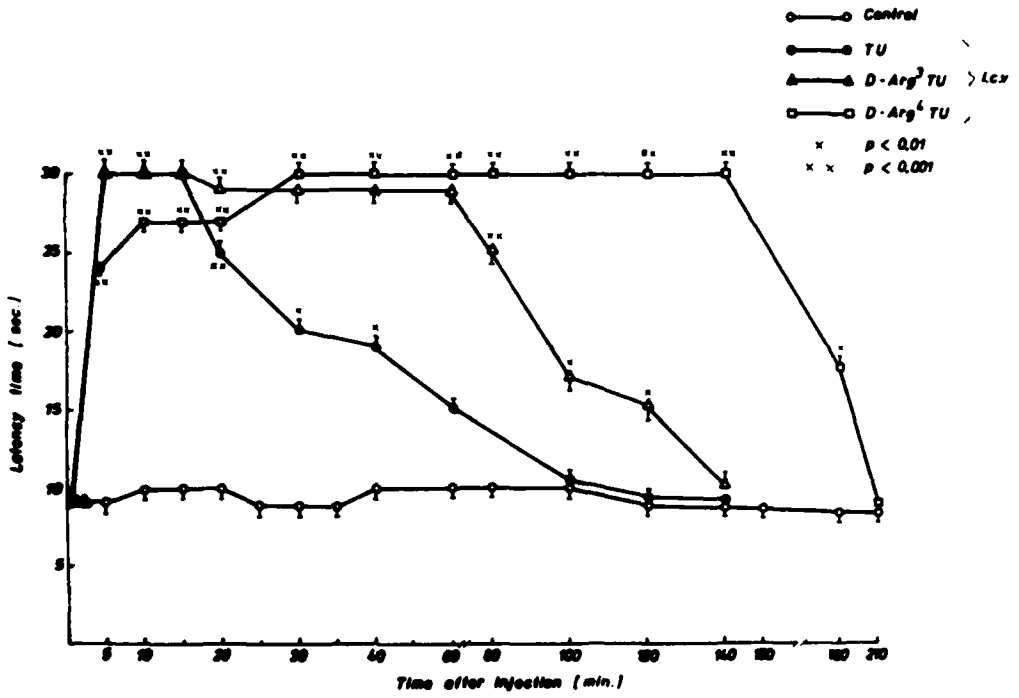

FIGURE 1. Antinociceptive effect of tuftsin (TU, 200 μg) and tuftsin analogues injected intracerebroventricularly (i.c.v.) in rats. The results are expressed as a mean of latency time of reaction on nociceptive stimulus ± SE; number = 10.

RESULTS

Antinociceptive Effect

Tuftsin administered i.c.v. in a dose of 200 μg induced an antinociceptive effect that lasted 40 min. (D-Arg^3)-tuftsin and (D-Arg^4)-tuftsin induced a stronger antinociceptive effect of longer duration (FIG. 1). (D-Arg^3)-tuftsin also induced abnormal

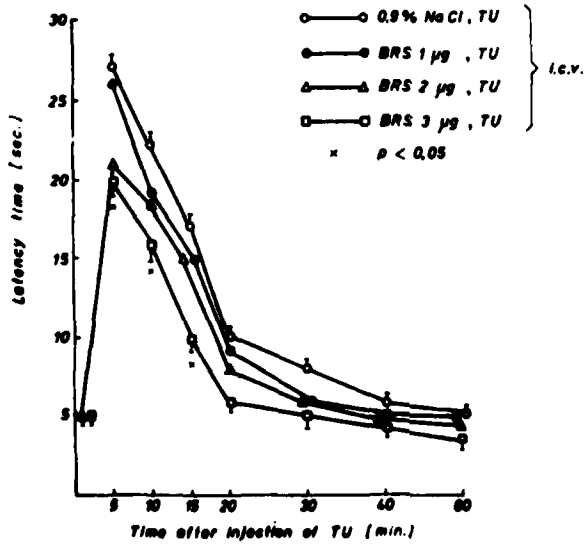

FIGURE 2. Effect of bradykinin (BRS) injected intracerebroventricularly (i.c.v.) 10 min before injection of 200 μg of tuftsin (TU) i.c.v. in rats. The results are expressed as a mean of latency time of reaction on nociceptive stimulus ± SE; number = 10.

behavior in the 10 min after injection; barrel rotations occurred (repeated rotation around the animal's longitudinal axis). This lasted for 30 min after injection.

Lys[4], D-Leu[1], and D-Leu[3]-tuftsin analogues induced a shorter antinociceptive effect, which persisted 20 min. This antinociceptive effect of tuftsin and its analogues was not affected by NAL (2.5 mg/kg i.p.) administered 30 min before the tuftsin injection. Val[1], D-Ala[3], D-Thr[3], D-Pro[3], D-Lys[2], D-Thr[1]-tuftsin analogues injected i.c.v. in a dose of 200 μg induced no antinociceptive effect. BRS (3 μg in rats, 2 μg in mice) injected i.c.v. 10 min before i.c.v. administration of tuftsin (200 μg) decreased the antinociceptive effect of this tetrapeptide (FIGS. 2 and 3).

Behavior

Tuftsin affected the motor activity of rats biphasically. It decreased exploratory activity of animals during the first 15 min after administration, then it increased this type of behavior between 60–90 min after injection (FIG. 4).

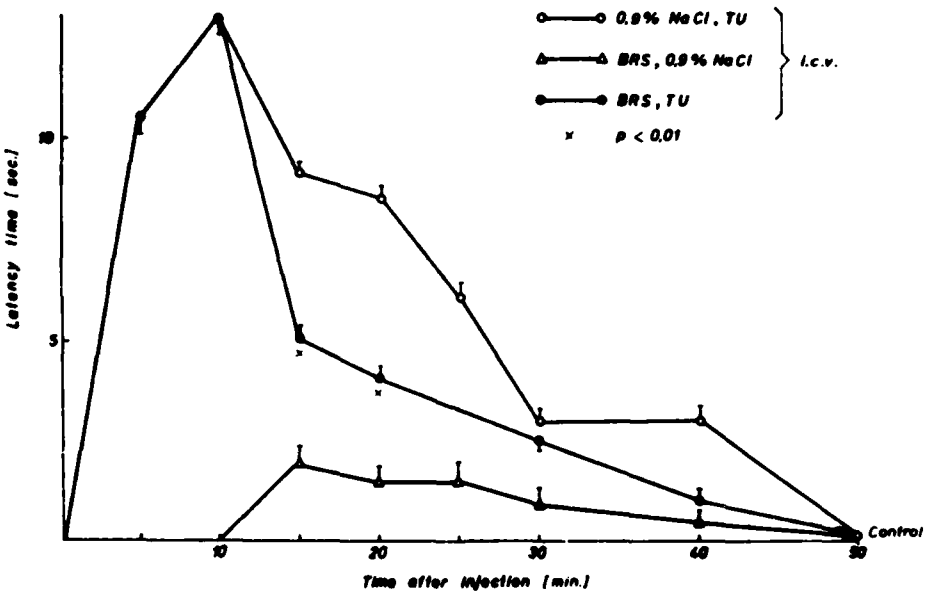

FIGURE 3. Effect of bradykinin 0.2 μg (BRS) injected intracerebroventricularly (i.c.v.) 10 min before injection of 200 μg of tuftsin (TU) i.c.v. in mice. The results are expressed as a mean difference of latency time of reaction on nociceptive stimulus between experimental mice and animals that received saline i.c.v. (control) ± SE; number = 10.

The locomotor activity of rats was at first diminished for about 15 min, then significantly increased 90 min after i.c.v. tuftsin administration (FIG. 5). These behavioral effects of tuftsin were not affected by NAL.

Cardiovascular System

Tuftsin increased the blood pressure of rats in a dose-dependent manner (FIG. 6). This effect was abolished by BRS but not by NAL (FIG. 7). Tuftsin decreased the rate

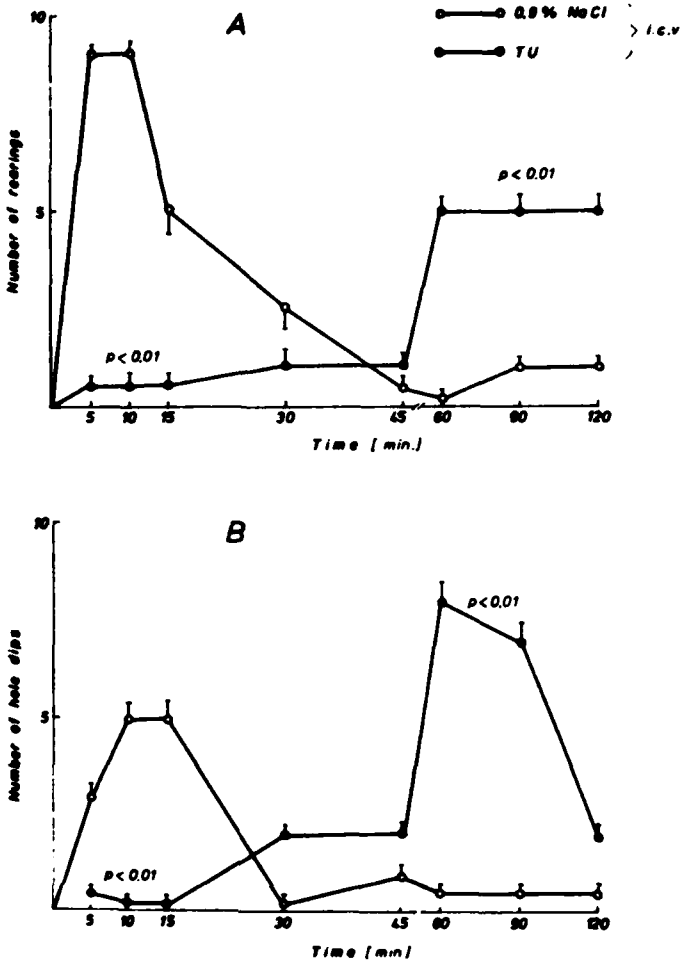

FIGURE 4. Exploratory behavior of rats after intracerebroventricular administration (i.c.v.) of tuftsin 200 μg (TU). Results are expressed as a mean of rearings (A) or hole dips (B) ± SE; number = 10.

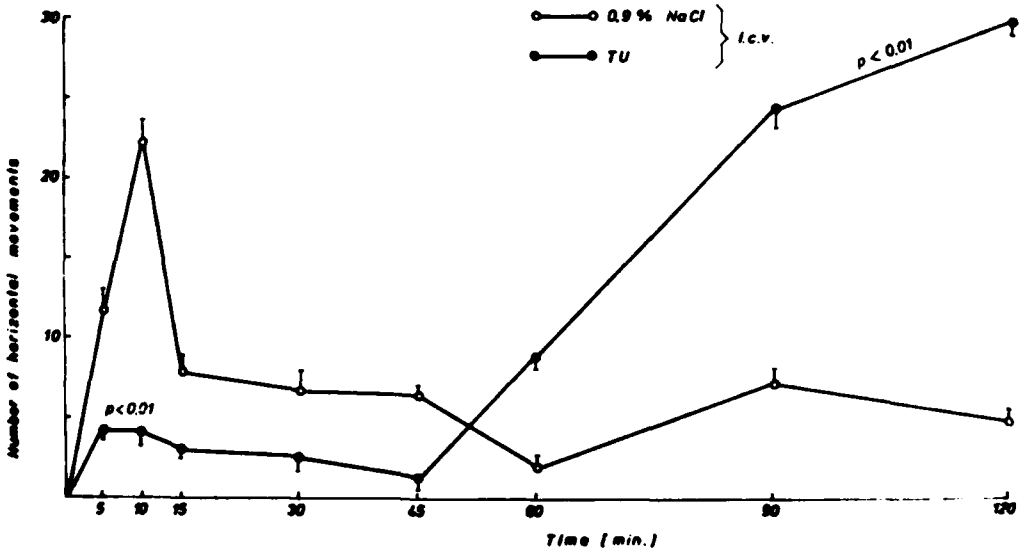

FIGURE 5. Locomotor activity of rats treated by 200 μg of tuftsin (TU) intracerebroventricularly (i.c.v.). Results expressed as a mean of horizontal movements ± SE; number = 10.

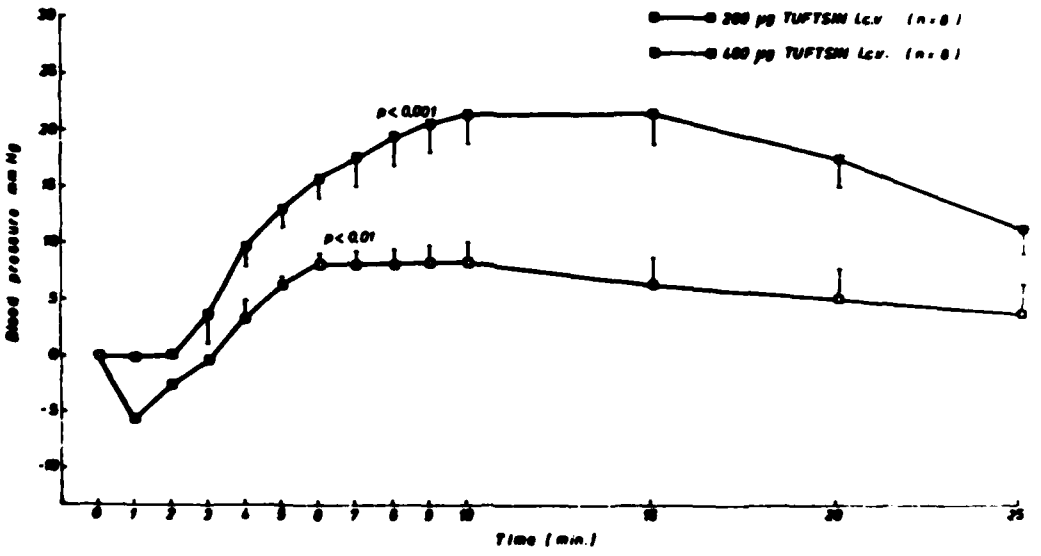

FIGURE 6. Influence of tuftsin injected intracerebroventricularly (i.c.v.), on the blood pressure of rats.

of respiration in rats in a dose-dependent manner (FIG. 8). This effect was not affected by BSR or NAL. Tuftsin had no effect on the heart rate.

DISCUSSION

Our results indicate that tuftsin, (D-Arg^3)-tuftsin and (D-Arg^4)-tuftsin have significant antinociceptive activity. We have not found in the literature any mention of similar observations. This activity is probably not mediated by opiate receptors, since opiate antagonist NAL has not affected the tuftsin antinociception. It is possible that

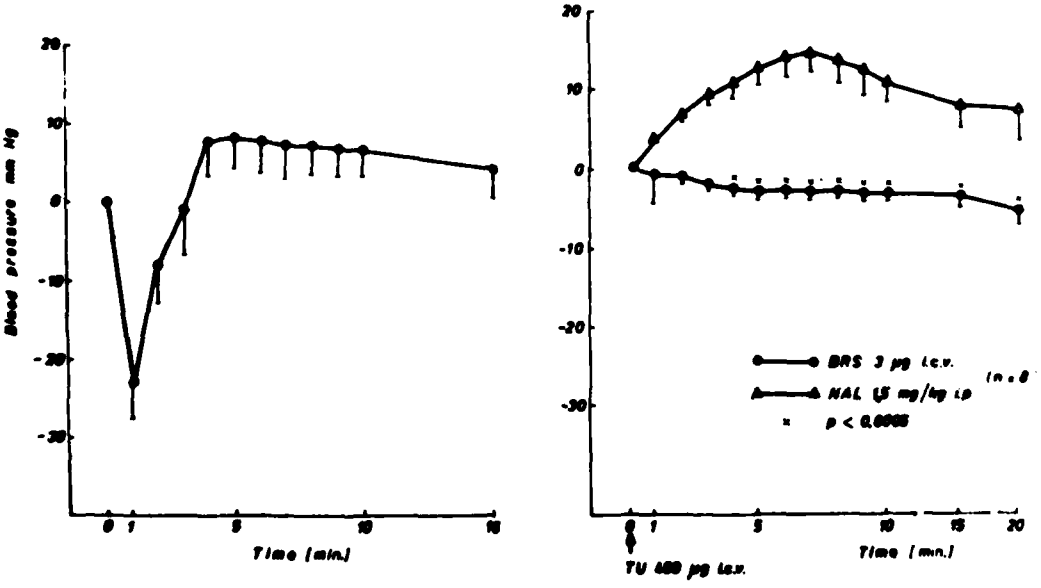

FIGURE 7. Influence of bradykinin (BRS) or naloxone (NAL) on blood pressure induced by intracerebroventricular injection (i.c.v.) of tuftsin (TU) in rats.

bradykinin might be involved in the mechanism of this phenomenon, since it antagonized the antinociceptive effect of TU in two animal species. There is no correlation between the potency of antinociceptive effects of tuftsin analogues and their potency on phagocytic activity. The relative phagocytic activity of tuftsin to its analogues was the following: D-Arg^4, 0.4; D-Arg^3, 0; D-Leu^3, 0; D-Thr^3 and D-Pro^3 were slightly active.[8] The abnormal behavior, the barrel rotations observed after (D-Arg^3)-tuftsin administration, was also described in rats after i.c.v. injection of somatostatin[9] or arginine-8-vasopressin and its analogues vasotocin and lysine-8-vasopressin.[10]

We have also shown that tuftsin affects exploratory and locomotor activity. These behavioral changes were biphasic. After a short depression, stimulation of the behavior was observed. Valdman *et al.*[11] have shown enhanced locomotor activity, induction of aggressiveness, and diminution of the acquisition of the passive avoidance reaction

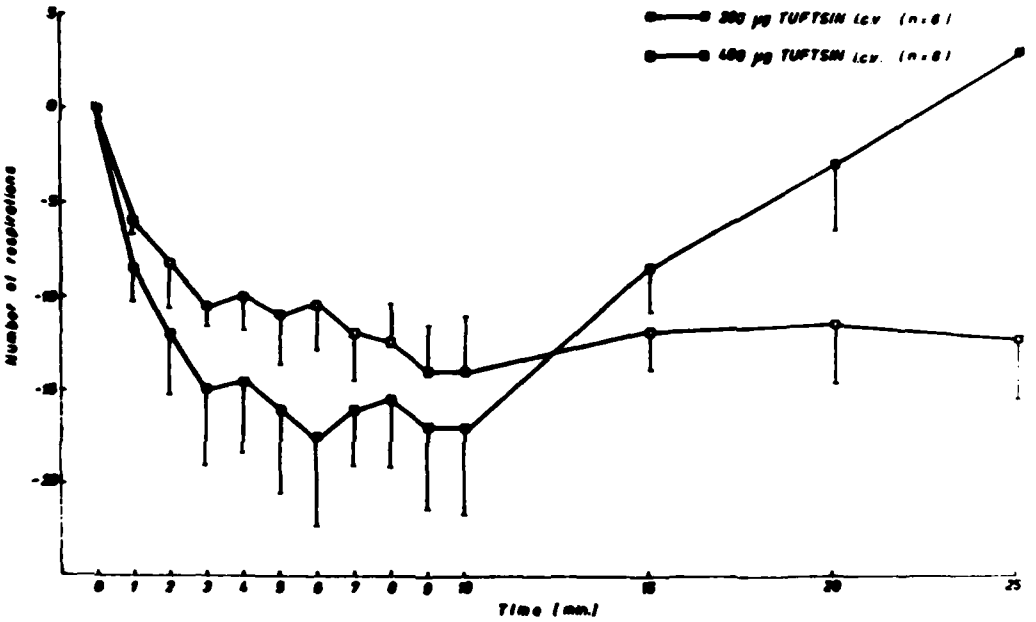

FIGURE 8. Effects of tuftsin administered intracerebroventricularly (i.c.v.) on the respiration of rats.

during a single reinforcement after administration of tuftsin in a dose of 500 mg/kg i.p. Later on, they have observed that tuftsin and (D-Arg^4)-tuftsin (20–250 mg/kg i.p.) decreased the immobility of mice under the experimental conditions of the despair test and increased exploratory activity in rats.[12] It is worth mentioning that (D-Arg^3)- and (D-Arg^4)-tuftsin have some similarity to the sequence of the basic fragment of α-neo-endorphin. Its NH_2-terminal fragment has a sequence: Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Arg-Pro.[13] The other central effect of tuftsin that we have observed was a dose-dependent increase of blood pressure in rats. This effect was abolished by BRS but not by NAL. Enkephalins administered intracisternally also induced in rats an increase in blood pressure, but this phenomenon was diminished by NAL.[14]

Tuftsin has a broad spectrum of biological activity summarized in recently published reviews[15–18] and in the work presented during this symposium. Tuftsin stimulates phagocytosis and pinocytosis, the motility of granulocytes, increases antibody formation, promotes bacterial killing properties and tumoricidal activity of phagocytic cells, and augments the immunogenic function of macrophages. Taking

into account the results presented here we believe there is evidence that the spectrum of biological activity of tuftsin is broadened by its activity in the central nervous system. Recently Smith and Blalock[19] suggested a circuit between the immune and neuroendocrine systems.

In conclusion, we suggest that:

1. Tuftsin has evident central effects.
2. Tuftsin and its analogues may represent a new class of substances of interest in the search for new analgesics.
3. It may be speculated that there exists some link between immunological processes and the functions of the central nervous system.

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The Influence of Tuftsin on Blood Pressure in Animals

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Unlike many other features of tuftsin's biological activity,[1] its influence on the circulatory system has not been so far specified in the literature. That such influence exists seemed probable, since tuftsin may be capable of liberating substances that affect the cardiovascular system from the cells possessing tuftsin receptors. Tuftsin may also have some effect on the vascular system acting indirectly through the central nervous system.

We have found that after the injection of tuftsin to guinea pigs and rats, there was a distinct increase of systolic blood pressure (SBP), and also diastolic blood pressure (DBP) of the animals. This effect, however, has not been observed in rabbits.

METHODS

The investigations were carried out on New Zealand albino rabbits, Wistar rats, and guinea pigs from Szczecin Medical Academy Farm. The animals were anesthetized with urethane injected intraperitoneally in a dose of 1 g/kg of body weight. Tuftsin (0.5% solution in physiological saline solution) was administered into the jugular vein of the animal. In the case of guinea pigs, doses of 0.15, 0.3, 0.5 and 1.0 mg, respectively, per kg of body weight were applied; in the case of rats and rabbits, only a dose of 1 mg per kg of body weight was used. Arterial blood pressure was measured by means of MCK-301 apparatus equipped with Nycotron transducer and registered on a N-338/AP recorder. Tuftsin was synthesized in the Institute of Chemistry of Wrocław University by the described method.[2]

RESULTS

Guinea Pigs

In these experiments, we used 15 male guinea pigs (650–700 g each). Each animal received a tuftsin injection 3–4 times. The reaction of the circulatory system appeared (with the exception of 2 cases) only after the first injection of the peptide. All animals responded to the injection with the varied increase of arterial blood pressure (SBP: 5–35 mm Hg; DBP: 5–25 mm Hg) appearing gradually after the injection. In 11 cases, the effect began after 3–7 min, in 4 cases after 10–30 min. At first, blood pressure increased gradually for 5–10 min (7 minutes average). The highest level of blood

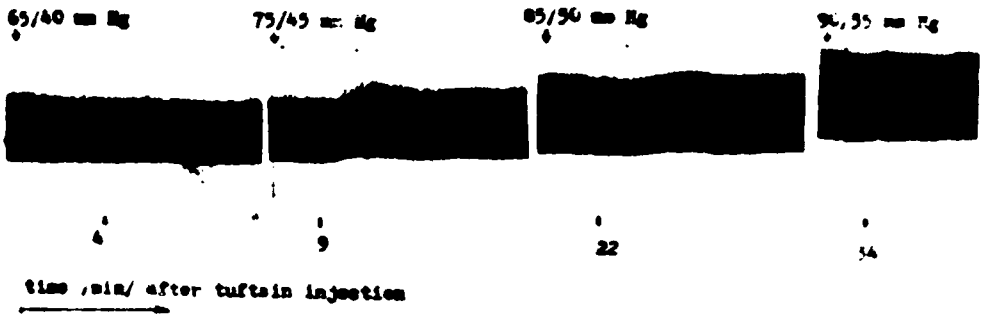

FIGURE 1. The increase of arterial blood pressure in guinea pigs after tuftsin injection (0.5 mg/kg of body weight).

pressure lasted for 5–40 min and then rapidly (3–6 min) decreased back to the initial value. The total duration of the effect differed depending on the animal (the range was 21–67 min). The changes of blood pressure evoked by tuftsin injection are exemplified in FIGURE 1. The moment of the beginning of the effect, its dynamics, and duration time did not seem to depend on the dose of injected tuftsin. The increases of SBP and DBP we observed after injection of various doses of tuftsin are shown in TABLE 1.

The lack of dependence between the blood pressure increase and the tuftsin dose induced us to combine into one group all the results obtained. For the whole group of animals, the increase of SBP was about 19 ± 8.3 ($p < 0.001$) and DBP 13 ± 7.3 ($p < 0.001$). As the initial blood pressure was relatively low (SBP 60–80 mm Hg, DBP 40–60 mm Hg), the relative SBP and DBP increases were significant (up to 60%); the average increase of DBP and SBP was about 30%. The injections of solutions of L-arginine, L-proline, L-lysine, and L-theronine, of adequate concentrations, did not evoke any change of arterial pressure.

Rats

In these experiments, we used 12 animals. The pressure reaction was similar to that observed in guinea pigs, but the observed responses of particular animals to tuftsin were more individualized than those of guinea pigs. The reaction appeared 4–12 min after the tuftsin injection; its duration time was 8–15 min. In one case, the duration time of the effect was 52 min. In this particular case the increase of the blood pressure was also very high (45 mm Hg for both SBP and DBP). From the whole group, two animals showed no blood pressure increase after tuftsin injection. The average increase

TABLE 1. Blood Pressure Increases in Guinea Pigs after Tuftsin Injection

Tuftsin Dose (mg/kg Body Weight)	Number of Animals	Increases of SBP (mm Hg)	Increases of DBP (mm Hg)
0.15	3	10; 10; 25	5; 10; 25
0.30	3	15; 20; 30	15; 15; 20
0.50	3	5; 5; 25	0; 5; 10
1.00	6	15; 15; 20	5; 10; 15
		25; 30; 35	20; 20; 20

of SBP was of about 18.8 ± 4.4 mm Hg ($p < 0.1$) and DBP of about 11.1 ± 3.4 mm Hg ($p < 0.2$).

Rabbits

Experiments on rabbits were performed with a group of 10 animals. Tuftsin was administered in doses of 1 mg/kg of body weight. In all cases, no pressure effect of tuftsin injection was observed.

DISCUSSION

The influence of tuftsin on the arterial pressure was distinct only in the case of rats and guinea pigs, no such effect appeared in the case of rabbits. As a rule, the effect appeared only once, that is, the second injection of tuftsin to the animal did not cause any subsequent change of its blood pressure. In all cases, the increase of blood pressure took place after a latency period of about 7–9 min. Considering these facts, it is probable that tuftsin does not directly influence heart or blood vessels, but causes the organism to liberate the other biologically active substances. It is very likely that the effects we observed are connected with the influence of tuftsin on the kallikrein-kinin or renin-angiotensin systems. The polymorphonuclear leukocytes are equipped with a variety of lytic enzymes exported to the interstitial space during phagocytosis (for a review see Ref. 3). Kinin activity is liberated from a human kininogen preparation containing plasminogen upon incubation with human polymorphonuclear leukocytes.[4] On the other hand, the recent results suggest that epithelioid cells in sarcoidosis, macrophages in leprosy, and Gaucher cells may actively synthesize angiotensin-converting enzyme (ACE). In the culture of monocytes, which are precursors of the above-mentioned cells, there takes place a 300-fold increase (as compared with *in vivo* conditions) of ACE production.[5] As we know, ACE plays the crucial role in bradykinin-angiotensin competition, because it is identical to kininase II, the enzyme that splits bradykinin.[6] The role tuftsin plays in blood pressure regulation processes may be connected with the stimulation of ACE release by the blood cells. Another possibility is that tuftsin or its degradation products inhibit the peptidases that participate in generation or biological degradation of angiotensin II. The effects we observed may also depend on the release of some other compounds, like histamine, from the cells. Still another possibility is that the pressure effects take place due to the influence of tuftsin on the central nervous system. According to data in the literature, tuftsin affects the central nervous system not only after intracerebroventricular administration[7] but also after intraperitoneal injection.[8]

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The Function of Tuftsin and Similar Sequences in Other Proteins

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The immunoglobulin molecule is a major component of the immune defense mechanism of many animal species. It possesses, it addition to a specific active binding site, the ability to regulate the activity, differentiation, and migration of cells. This is achieved by interacting either with various soluble components in the fluids, such as complement,[1,2] or directly with specific cell-surface receptors capable of recognizing a defined stereospecific conformation of the Ig molecule (reviewed in ref. 3).

The investigation of the molecular basis of these interactions revealed that all these activities were confined to the evolutionarily preserved constant region of the Ig molecule (Fc). The specific recognizing receptors for these determinants are defined as Fc receptors. These receptors, through which the Ig molecule exerts its regulatory function, are found on many cell types. Thus, one can find such receptors on almost all phagocytic cells, T cells, B cells,[3] NK cells,[71,72] tumor cells and many virally infected cells. Signals exerted by Fc-associated determinants through these receptors may lead to the elicitation of effector functions exerted by such cells and to their clonal expansion and differentiation. These functions can be performed either by free Ig molecules, or their isolated Fc fragments, or by antigen-antibody-activated, conformationally changed immune complexes. This may explain the immunoregulatory functions of immune complexes and the mechanisms by which these complexes with complement cause severe injuries to different tissues and organs, thus being responsible for diseases invoked by immune complexes.[6] In an effort to elucidate the regulatory mechanism by which the Fc portion exerts its effector functions, it was found that this fragment or several peptides derived from this portion may regulate the proliferation and effector functions of macrophages and T cell-dependent B cell activation.[7-12]

The ability of Fc fragments to regulate the proliferation and differentiation of lymphocytes is not exclusively confined to B cells, as found by Berman *et al.*[7,8] and Morgan and Weigle.[9-12] It may also regulate the proliferation, differentiation, and Fc-receptor formation of additional subsets of lymphocytes, that is, T lymphocytes. It was thus demonstrated that the Fc portion of IgA may cause an increase in the frequency of specific subsets of T cells displaying the T-alpha receptor. These cells are involved in the regulation of IgA synthesis[13] and in the increased density of Fc receptors for IgA on these cells.[14] A significant number of these Fc receptors may be shed in the vicinity of these cells. This finding gains further significance in view of the

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recent observation of Löwy *et al.*[15] that freely secreted Fc receptors may specifically suppress the activation of isotype-specific antibody-producing cells, in accordance with the isotype-specific Fc receptor used thus regulating B cell differentiation. Furthermore, it was demonstrated[16] that the activated $Fc\gamma_{2b}$ receptor of the murine macrophage cell line $P388D_1$, may by itself possess phospholipase A_2 activity. This may be relevant to the mechanism by which the Fc receptor displays its functions in ADCC-functioning cells and cells that display tumoricidic and parasiticidic Fc-induced activities at the level of the membrane.

Thus, the constant fragment of the antibody molecule is involved in the regulation and activation of different cascades of important biological functions. Yet, numerous studies have been performed to elucidate the genetic basis of the formation and generation of Ig diversity whereas only relatively limited effort was directed to the understanding of the molecular basis by which the Fc fragments display their function. New and promising opportunities have been provided by the original studies initiated by Najjar and coworkers when investigating the physiological role of cytophilic gammaglobulins.[17-22] They demonstrated that fractions of the immunoglobulins from mammalian origin, obtained through chromatography on phosphocellulose columns, have selective affinities to different blood cells: the erythrocyte,[18,22] the thrombocyte[4,22] and the granulocytic leukocyte.[17,19,22]

Further studies on the leukophilic gammaglobulin fraction, leukokinin, which stimulates the phagocytic activity of polymorphonuclear leukocytes, revealed that the whole activity of leukokinin could be attributed to a small, daughter peptide, derived from the protein and characterized as L-threonyl-L-lysyl-L-prolyl-L-arginine.[5,22-24] This basic tetrapeptide was named tuftsin, and it has been chemically synthesized.[23,25] A complete identity in physicochemical and biological features of both the natural and synthetic compounds was established.[23-25] The relevance of tuftsin to the clarification of Fc-mediated functions was established by a series of studies that demonstrated that this tetrapeptide is located in the CH2 domain of the Fc fragment of the gammglobulin molecule[26] occupying positions 289–292 of this chain:

....His-Asn-Ala-Lys-Thr-Lys-Pro-Arg-Glu-Glu-Glu

289 292

These studies revealed that the release of the active tetrapeptide is performed by two successive cleavage reactions by splenic and leukocytic enzymes. The two-step cleavage is reminiscent of the production of angiotensin.[27,28] The evidence for the revelance of tuftsin to Fc functions is the clear demonstration that this tetrapeptide is capable of activating polymorphonuclear, mononuclear phagocytic, and nonphagocytic cells to perform effector functions that are known to be induced by the Fc fragment of Ig. Thus, tuftsin is capable of: (1) enhancing phagocytosis; (2) activating the hexose-monophosphate shunt involved in bacterial killing, due to the formation of toxic superoxide and hydroxyl radical; (3) enhancing specific antigen presentation by macrophages for the education of antigen reactive memory T cells; (4) regulating *in vivo*-induced immune responses to thymus-dependent and thymus-independent proteins; (5) increasing inflammatory cell migration; (6) increasing the *in vivo* and *in vitro* antibacterial activity performed by phagocytic cells; and (7) potentiating antitumor activities (reviewed in ref. 29).

It was thus established that the CH2-originating tuftsin peptide displays many functions usually performed by the Ig Fc fragment. An obvious sequential step aimed at understanding the mode of action of the regulatory functions was a careful structure-function-related analysis. Indeed, such studies have been performed, using various synthetic analogues with defined modifications of the various possible active

groups of tuftsin by Fridkin, Konopińska, and Nishioka (reviewed in refs. 29, 30). Taken together, the functional studies performed on different systems and the conformational energy studies by Fitzwater *et al.*,[31] and neglecting some minor discrepancies, one finds that characteristic structural requirements are needed for functional regulatory integrity. In general, it seems that four structural "working rules" should be considered if significant biological activity is to be anticipated from a tuftsin analogue: (1) the overall positive charge and its distribution within the peptide should be kept as close as possible to that of tuftsin; (2) a proline residue must be implanted in close proximity to and ideally between the two positive charges; (3) the integrity of the NH_2-terminal threonine residue should be maintained, and (4) chemical modifications such as chain extension will only be at the COOH-terminal of tuftsin.[29]

These strict structural requirements displayed by tuftsin and the known fact that receptors for the constant portion of Ig are membrane-associated have suggested the existence of a specific membrane receptor for tuftsin. Although it is not yet certain whether the Fc receptor and the receptor for tuftsin are identical, studies performed by Stabinsky *et al.*,[32] Bar-Shavit *et al.*,[33] and Nair *et al.*[34] have clearly demonstrated the existence of specific cell receptors for tuftsin on polymorphonuclear cells, monocytes, macrophages and lymphocytes. These cells display approximately 50,000–100,000 binding sites per cell, with an equilibrium dissociation constant ranging between Kd 1.3×10^{-7} and 5.3×10^{-8} *M*. Comparing the minimal structural identity required for binding to these active sites and activities performed by tuftsin, a high degree of overlapping similarities were observed, indicating that many, if not all, functions performed by tuftsin are initiated through interactions with these receptors. Furthermore, Gottlieb *et al.*,[35] using fluorescent intensification analyses, revealed that the regulation and redistribution pattern of tuftsin receptor sites of macrophages appears to follow patterns previously described for growth factors and other peptide hormone-receptor complexes.[36,37]

Considering just the phagocytosis-potentiating effect of tuftsin, one could assume that the main function of tuftsin resulting from the interaction with its receptor is the induction of processes that lead to the perturbation of the cytoplasmic membrane, thus permitting endocytic events. But, surveying all the other efects of tuftsin, it appears that these effects are achieved through major changes within the cell and its membrane. These should involve the activation of numerous enzymatic pathways, cytoskeletal structure changes and various differentiation steps. Hence, tuftsin is likely to induce changes characteristic of other differentiation-inducing peptides and proteins, that is, the redistribution in different cellular compartments of calcium concentrations and induction of changes in the relative concentration of regulating cyclic nucleotides. Indeed, Stabinsky *et al.*[38] demonstrated that tuftsin induces an increase of cyclic GMP levels of tuftsin-activated polymorphonuclear cells, accompanied by a decrease in cyclic AMP levels. They also demonstrated that tuftsin releases $^{45}Ca^{2+}$ from polymorphonuclear cells preloaded with the isotope. These results suggested that modulation of both the amount of cell-associated $^{45}Ca^{2+}$ and the intracellular levels of cyclic nucleotides are "by-products" in the mechanism by which tuftsin displays its regulatory functions. Furthermore, in our laboratory we have found that tuftsin induces *de novo* synthesis of both RNA and proteins in peritoneal exudate macrophages.

These results indicate that tuftsin shares many characteristics with other peptide hormones and growth factors. Furthermore, investigating structure-function relationships of tuftsin, we and others noticed that the dipeptide prolyl-arginine (Pro-Arg) and the "anti-allergic peptide" have a considerable stimulatory action. The Pro-Lys sequence in which a similar positively charged amino acid is located next to Pro is

probably also recognized by the receptor as can be inferred from the tuftsin-like activities of Thr-Lys-Pro-Lys.[39] The receptor seems to possess some affinity to the reverse sequences Arg-Pro. Thus, it seems plausible that the minimal determinative requirements for the specific binding and activity of tuftsin are dependent on the amino acid couple Pro-Arg. Pro-Lys and Arg-Pro are also recognized by the receptor but with a lower affinity. This possibly stems from the fact that a positively charged amino acid is forced by Pro into a negative locus within the receptor site. Proline is unique in its formation of a secondary amide peptide bond, which has particular structural-conformation consequences. In addition to its structural role, the Pro may determine the protection of the peptide's active epitope against degradation by nonspecific enzymes.[40] Since tuftsin possesses typical hormone-like activities, and in view of the minimal structural requirements for its function, one may raise the question of whether tuftsin is a unique hormone-like peptide inserted for specific functions in the constant fragment of Ig. Comparing the primary structure of various regulatory hormones, we were surprised and impressed by the common abundance of the Pro-Arg, Arg-Pro and other tuftsin-like sequences. Thus, one finds similar sequences in the following biologically active peptides and proteins (TABLE 1).

TABLE 1. Occurrence of Pro-Arg, Pro-Lys, and Arg-Pro Sequence in Some Biologically Active Peptides[41]

Peptide[41]	Sequence
Arginine-vasopressin	-Pro-Arg-Gly-NH_2
"Anti-allergic peptide"[42]	H-Asp-Ser-Asp-Pro-Arg-OH
Neurotensin	-Lys-Pro-Arg-Arg-Pro
Adrenocorticotropin hormone	-Arg-Arg-Pro-Val-
Luteinizing hormone-releasing hormone (LH-RH)	-Leu-Arg-Pro-Gly-NH_2
Substance P	H-Arg-Pro-Lys-Pro
Bradykinin	H-Arg-Pro····-Pro-Phe-Arg-OH
Lysine-vasopressin	-Pro-Lys-Gly-NH_2
Melanocyte-stimulating hormone	-Pro-Pro-Lys-
Insulin B chain, human	-Pro-Lys-Thr-OH
Pancreatic polypeptide[43]	-Thr-Arg-Pro-Arg-

The similarity existing between tuftsin and tuftsin-analogous sequences, which seem to be abundant in several bioregulatory peptides, is not deduced only from the shared identical amino acids at certain positions. Functional assays showed that (1) some of these peptides like LH-RH, bradykinin, "anti-allergic peptide," substance P, and neurotensin may exert tuftsin-like activation of the phagocytic and antigen-presenting functions of phagocytic cells;[39,44,45] (2) substance P and neurotensin were shown to compete efficiently and specifically with tuftsin on tuftsin-binding sites;[39,44,45] and (3) tuftsin showed 20% of the myotropic activity exerted by angiotensin on the isolated ascending colon of the rat.[46] Thus, the possibility exists that tuftsin is not a unique Ig-associated regulatory peptide, but is related to an ancient common ancestral gene which, during evolution, yielded different Pro-Arg-containing regulatory peptides that act on different target cells sharing at least in part common membrane-associated signal-generating systems. This assumption gains support from three independent studies[47-49] analyzing the binding of neurotensin and adrenocorticotropin hormone and their segments.[49] They suggest that Arg-Pro and Pro-Arg determinants are directly associated with the binding and activity of these hormones. These findings, although

indicating a possible common structural relationship for several previously unrelated regulatory peptides, do not elucidate the yet unknown mode of action of these peptides. Yet, several other findings may contribute to the clarification of this question. Thus, analyzing the sequence of several phosphoproteins with affinity to nucleic acids revealed a partial sequence homology between the murine leukemia virus (R-MuLV), derived p12 gag phosphoprotein and histone H5 isolated from goose and chick red blood cells.[50] Even more intriguing is the fact that both p12 and H5 contain the complete sequence of tuftsin and the (Ala[1])-tuftsin.[50] The presence of tuftsin-like sequences is not unique to H5, but similar sequences were also found, as reported by Konopinska *et al.*,[51] in other histones. They demonstrated that peptides with tuftsin-like activity can indeed be isolated by enzymatic degradation of histones. In addition, Luftig *et al.*[52] and Suk *et al.*[53] demonstrated that tuftsin is capable of regulating the split of the p65–70 gag gene product and enhances MuLV virion budding and production.

Other major findings that may be relevant to the function of Pro-Arg-containing regulatory peptides are those of Nimmo *et al.*[54,55] They demonstrated the existence of an inhibitor of a phosphoprotein termed inhibitor-1. Inhibitor-1 inhibits the phosphorylase-phosphatase, phosphorylase-kinase-phosphatase, and glycogen-synthetase-phosphatase activities of protein phosphatase-1. Recently, phosphatase-1 was found to be present at very similar concentrations in a wide variety of mammalian tissues, even in those tissues were glycogen metabolism is of very minor importance. Evidence is accumulating that this enzyme may dephosphorylate many proteins involved in regulating a variety of metabolic processes. It is therefore possible that inhibitor-1 will be found to participate in the regulation of other cellular events that are controlled by cyclic AMP. This implies that the tissue distribution of inhibitor-1 follows that of protein-phosphatase-1.

The amino acid sequence at the phosphorylation site of this phosphoprotein (inhibitor-1) is Ile-Arg-Arg-Arg-Arg-Pro-Thr(P)-Pro-Ala-Thr, a sequence that resembles a tuftsin analogue sequence.

Analyzing the various tuftsin analogues and their optimal concentrations for the activation of various phagocyte activities, including antigen presentation, certain discrepancies emerged: (1) Increasing the concentrations of tuftsin and similar analogues leads to a steady increase in phagocytic activity until a typical saturation level is reached. This is not the case when concentration-related activity is tested in antigen presentation. In this case, lower concentrations of tuftsin are needed and following a rapid increase, an optimum is reached followed by a quick fall in activity. (2) (Ala[1])-tuftsin, which is a potent activator of antigen presentation, is an inhibitor of phagocytosis and of activation of phagocytes by LPS.[29] Furthermore, (Ala[1])-tuftsin inhibited nitroblue tetrazolium reduction and was inactive in the glucose oxidation assay. Thus, a discrepancy exists between the structural requirements for the activation of phagocytes and those associated with more complicated differentiation pathways, in which (Ala[1])-tuftsin seems to be more active. (3) Examining competition profiles of tuftsin binding and activation by neurotensin on macrophages, the apparent existence of high- and low-affinity tuftsin binding sites is suggested.[44] (4) Studies of concentration and activity of the chemotactic peptide N-formyl-Met-Leu-Phe showed a biphasic concentration-dependent behavior of phagocytes. At low concentrations migration is maximal, and at high concentrations, migration is inhibited and a "firing off" of effector constituents is initiated.[56]

On the basis of these observations and discrepancies, we would propose the following: these cells, probably at their early stages of differentiation already possess two different receptors for tuftsin with different affinities. The interaction of tuftsin with one receptor causes the induction of short-term, rapidly occurring signals at the

level of the membrane, resulting in membrane perturbation, phagocytosis, and elicitation of effectors capable of killing microorganisms and tumor cells. Following this interaction, the receptor-tuftsin complex will be endocytized, leading to "down regulation" and final degradation of tuftsin. The interaction of tuftsin with a second tuftsin receptor will trigger events similar to those triggered by growth factors and hormones. These include the induction of early signals, generation of a "coated pit" and migration of the newly formed receptosome into cytoplasmic compartments, possibly to the nucleus. As a result of this, tuftsin may be released at different sites including the nucleus and then, similar to inhibitor-1, may regulate the activity of different protein phosphorylation events, including histones. Because of the high affinity to nucleic acids, it may act as a phosphopeptide phosphorylated at the Thr residue to interact with nucleic acids. It may thus influence processes of gene expression or gene amplification, regulating mainly processes of differentiation. (Ala^1)-tuftsin may be more specific for the second receptor rather than for the first. These receptors may appear in different concentrations on different phagocytic cells and may vary in concentration during different stages of differentiation. The suggestion of the existence of two different receptors for the same ligand regulating different activities of a mammalian cell is supported by findings concerning B lymphocytes. These were shown to possess two different receptors, that is, membrane-bound IgD and IgM, whose interaction with the same ligant leads to completely different differentiation stages of the B lymphocyte.[57] This proposed model may lead to a new approach, not only to the function of tuftsin, but also to other bioregulatory peptides and proteins in the function of which a tuftsin-analogous sequence plays a role.

Sequence analysis of the proteins and their coding genes demonstrated that additional regulatory peptides, proteins and glycoproteins that control cell differentiaion and different immune functions evolved during evolution by gene duplication and mutations from a common, Ig-related, ancestral gene. This gene is coded for a single domain of approximately 110 amino-acid residues found in the Fc portion of Ig molecules.[58] It seems that this ancestral gene contributed to the evolution of: (1) functional domains of the heavy chain of histocompatibility antigens, beta-2 microglobulin, the alpha and beta chains of glycoproteins of the I region in the mouse and DR region in man;[60-62] (In these chains, the Ig-related domains are found in close proximity to the outer surface of the cytoplasmic membrane.) (2) Thy 1 (rat brain antigen); (3) the male histocompatibility antigen; Hy; and (4) the thymus-derived hormone thymopoietin, which regulates the differentiation of T lymphocyte stem cells.[58] The question then arises whether during evolution from this ancestral Ig-related gene, which is involved in regulatory functions, the tuftsin-like sequences were also preserved. Indeed, one finds in the thymopoietin sequence, at positions 11-14, the tetrapeptide Thr-Lys-Glu-Lys. Similarly, one finds the sequences Thr-Gln-Pro-Arg in positions 483–486 of the IgE heavy chain and at positions 233–236 the sequence Thr-Arg-Pro-Ala in the heavy chain of HLA-B7.[58] Similar sequences are found in $H\text{-}2K^b$, $H\text{-}2D^d$ and $H\text{-}2D^b$ [59] encoded by the analogous MHC system of the mouse. Furthermore, activated lymphocytes and leukocytes were shown to secrete various antigen-specific and nonspecific "helper" and "suppressor" factors,[63-66] and molecules that stimulate growth and differentiation of granulocytic progenitor cells.[67]

Immunochemical analysis of these factors has revealed that they contain cross-reacting structures with beta-2 microglobulin and MHC-encoded glycoproteins.[68]

In summary, the analysis of the function of tuftsin and tuftsin-like sequences containing molecules as outlined in our present review suggests a basis for the understanding of the evolution and function of many regulatory molecules such as peptide hormones, Ig, partially Ig-related major histocompatibility complex-encoded glycoproteins, and similar structure-related glycoproteins that in their free, soluble

form regulate the activity and differentiation of various target cells. The regulatory part of these molecules may have evolved from a highly conserved, common ancetral gene. One may question whether such an assumption is justified just on the basis of the abundance of a tetrapeptide in different proteins, a sequence that could have arisen at random. It appears to us, however, that the probability that a tuftsin-analogous sequence will appear by chance is beyond statistical probability. Furthermore, thus far, of hundreds of known sequences of proteins, the tuftsin sequence or its functional analogues were found only in bioregulatory molecules that regulate cell activity following recognition by specific binding sites. This sequence was not observed so far in either structural proteins or proteins possessing enzymatic activities.

ADDENDUM

It would be hard to conclude the survey of tuftsin- or tuftsin analogue-containing molecules without mentioning three additional important proteins that contain analogous sequences. These are: (1) the influenza hemagglutinin JAP HA (H2."57), residues 214–217 are occupied by a close analogue Thr-Arg-Pro-Lys;[68] (2) procollagen type I, *p*-alpha I(I), the sequence Thr-Gly-Pro-Arg[69] is found in the COOH-terminal end of the peptide that is removed when procollagen is converted to collagen; and (3) another Ig-constant, region-related protein, the COOH-reactive protein (C.R.P.) that contains the sequence Lys-Pro-Arg and twice the Thr-Lys-Pro sequence.[70]

The presence of such sequences of these molecules may be of significance to relevant inflammatory processes because the hemagglutinating components of viral coats are usually involved in the activation of NK cells.[71,72] The degradation of collagen molecules by various microorganisms and tumor cells is known to produce chemotactic factors that have not yet been analyzed. COOH-reactive protein[70] (C.R.P.) is an acute inflammatory protein that in the presence of calcium interacts specifically with the Fc receptors, fixes complement, and by nonspecific bindings to various microorganisms, may serve as a first-defense opsonizing molecule. It thus performs many of the regulatory functions performed by the related Fc portion of the Ig.

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Immunopharmacological Properties of Tuftsin and of Some Analogues

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INTRODUCTION

Since the description by Najjar and Nishioka[1] that the basic tetrapeptide (Thr-Lys-Pro-Arg), originating from a leukophilic IgG fraction, stimulates the phagocytic activity of polymorphonuclear leukocytes (PMN) and macrophages, many works have been devoted to the analysis of the biological properties of this peptide. It appears from these studies that all functions and all metabolic activities of PMN and macrophages that have been examined were stimulated by tuftsin, present in hormone-like concentrations, through binding to specific cellular receptors. Among them, the immunogenic function of macrophages by antigen presentation to T lymphocytes was shown by Tzehoval *et al.*[2] to be enhanced after *in vitro* treatment with tuftsin. As macrophages play many other roles in the regulation and the expression of immune functions, either directly or by interacting with lymphocytes, it can be asked whether tuftsin could influence the immune reactivity of the host, and in this way could act as a physiological immunomodulator. This hypothesis finds additional support in the demonstration by Nair *et al.*[3] of the presence of tuftsin receptors on lymphocytes suggesting that these cells may be directly stimulated by this peptide.

In the present work, we investigated the effect of tuftsin administration to immunocompetent mice on the expression of various humoral and cell-mediated immune responses. Stimulatory or inhibitory effects were obtained depending on the dose administered and on the time of administration.

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In the second part of this study, we have examined the *in vitro* effects of tuftsin on two PMN functions: chemotactic and phagocytic activities. This was done in parallel on normal PMN and inflammatory PMN in order to determine whether the physiopathological state of the target cell could influence its reactivity to tuftsin. We also looked for modifications of the phagocytic and tumoricidal activities of macrophages.

Disposal of tuftsin analogues is important, firstly in order to establish structure-activity relationships. Secondly, it can be expected to obtain molecules that may be more resistant to degradation than the native peptide. Thirdly, other types of molecules can be coupled to tuftsin in order to amplify its activity. Martinez *et al.*[4] presented the rationale for the synthesis of tuftsin analogues characterized by the replacement of threonyl in position 1 by another amino acid or by coupling a molecule of indomethacin (Indo) to tuftsin or to (*des*-Thr[1])-tuftsin. In the third part of this work, these analogues were compared to tuftsin for their stimulatory activity of macrophage phagocytosis and of natural killer (NK) cell activity after *in vivo* administration to mice as these two mechanisms play an important role in the defenses against bacteria and tumor cells.

RESULTS

In Vivo *Immunomodulating Activity of Tuftsin*

In order to investigate whether tuftsin could act as an immunomodulator, it was administered in a single i.v. or i.p. injection to 8–12-week-old female (C57BL/6 × DBA/2)F1 hybrid mice in a dose of 25 μg/animal, unless otherwise stated. Various immunological tests were performed from day 0 to day 14 after the treatment. The preparation of tuftsin used was obtained by synthesis as already described.[4,5]

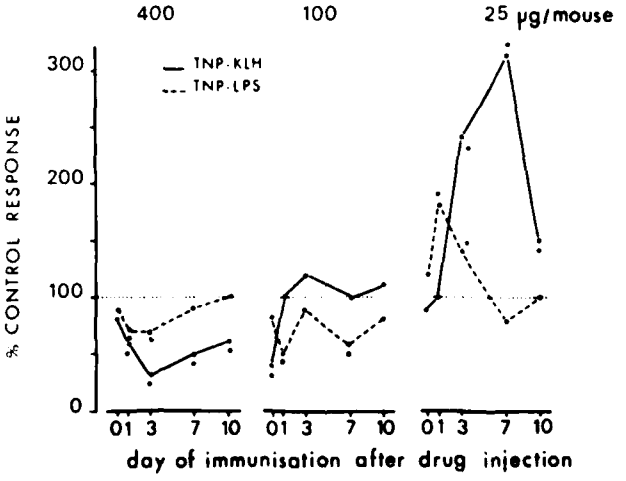

FIGURE 1. Effect of a single i.v. injection of various doses of tuftsin on the antibody response to T-dependent (TNP-KLH) or T-independent (TNP-LPS) antigens. Control and tuftsin-treated mice (10 animals/group) were immunized on day 0 with either 300 μg TNP-KLH or 0.3 μg TNP-LPS in saline and an assay for anti-TNP antibody response was performed, respectively, 4 and 3 days later. Results are expressed as the percentage of the mean number of IgM antibody-forming cells/spleen in control mice. * indicates that the response of tuftsin-treated mice is significantly different ($p < 0.05$) from control response.

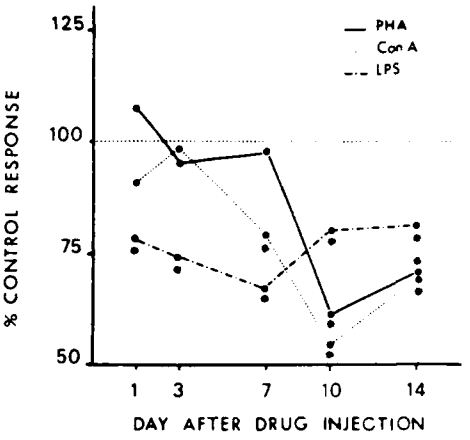

FIGURE 2. *In vitro* proliferative response of spleen cells to T-cell (PHA, Con A) or B-cell (LPS) mitogens at various times after a single i.v. injection of 25 μg tuftsin. Spleen cells (2.5×10^6/ml) from control and tuftsin-treated mice were incubated for 48 hours with PHA (1/500e), Con A (2 μg/ml) or LPS (100 μg/ml) and [^{3}H]-TdR was added during the last 4 hours. Results are expressed as the percentage of [^{3}H]-TdR incorporation by control spleen cells. * indicates that the response of tuftsin-treated spleen cells is significantly different ($p < 0.05$) from control response.

Effect of Tuftsin Administration on Antibody Responses

Tuftsin was injected i.v. in doses of 400, 100, and 25 μg/mouse at various times before immunization with either 300 μg trinitrophenylated-hemocyanin (TNP-KLH) as a T-dependent antigen or 0.3 μg trinitrophenylated-lipopolysaccharide (TNP-LPS) as a T-independent one. The number of anti-TNP antibody-forming cells in the spleen was determined at the peak of the antibody response, that is, 3 days and 4 days after TNP-LPS and TNP-KLH immunization, respectively.

As shown in FIGURE 1, when injected in a dose of 400 or 100 μg/mouse, tuftsin induced a depression of T-dependent and T-independent antibody responses at various times after its administration. Up to a 70% reduction in the number of antibody-forming cells/spleen could be observed. Decreasing the dosage to 25 μg/mouse resulted in the appearance of a stimulatory effect. The response to TNP-KLH was enhanced to a greater extent and for a longer time after tuftsin injection than the response to TNP-LPS, suggesting that the mechanism of the stimulatory activity of tuftsin was different for each type of response. Additional experiments in which 100 μg to 10 μg of tuftsin were administered have confirmed that 25 μg/mouse maximally potentiated the antibody response to TNP-KLH (results not shown). This dosage was therefore used in all the following experiments.

Effect of Tuftsin Administration on Spleen Cell Mitogen Responsiveness

Spleen cells from tuftsin-treated mice were examined for their proliferative response to *in vitro* stimulation with either phytohemagglutinin (PHA) or concanavalin A (Con A) as T cell mitogens, or with lipopolysaccharide (LPS) as a B cell mitogen. As shown in FIGURE 2, the response to LPS was moderately inhibited (20 to 33% reduction in [^{3}H]-TdR incorporation) during the 14-day period following tuftsin administration. The response to T-cell mitogens remained unaffected 1 and 3 days after tuftsin injection but was maximally depressed on day 10 with a 39% and 46% inhibition of ^{3}H-TdR incorporation in spleen cells stimulated with PHA and Con A, respectively.

This impaired mitogen responsiveness did not appear to result from a stimulation of nonspecific suppressor cells. Spleen cells from tuftsin-treated mice did not significantly inhibit the proliferative response of normal spleen cells to T or B cell mitogens in

coculture experiments whether they were mixed in 1:2, 1:1 or 2:1 ratios (results not shown).

Effect of Tuftsin Administration on T Cell–Mediated Cytotoxicity

Spleen cells from tuftsin-treated mice and from normal mice were compared for their ability to differentiate into cytolytic T lymphocytes (CTL) upon *in vitro* immunization with allogeneic tumor cells. In this experiment, C57BL/6 mice were injected i.v. with tuftsin, and their spleen cells, harvested at different times after the treatment, were cultured for 5 days in the presence of mitomycin-treated P815 mastocytoma cells of DBA/2 origin. Their cytotoxic activity against ^{51}chromium-labeled P815 tumor cells was thereafter assessed in a 4-hour chromium-release test using different effector-to-target-cell ratios. This allowed us to determine the value of the lytic unit 30, which is the number of effector cells required to lyse 30% of the target cells. As shown in FIGURE 3, a slight but significant augmentation of CTL activity was observed with spleen cells coming from mice given tuftsin one day before *in vitro* immunization. This was reflected by a 30% increase in the number of lytic units/10^7 immunized spleen cells. At longer time intervals after tuftsin injection, spleen cells exhibited a decrease in their capacity to differentiate into CTL, which was maximal on day 7 as shown by a 50% reduction in the number of lytic units/10^7 cells.

Effect of Tuftsin Administration on Interleukin 2 (IL 2) Production

It is now well established that the *in vitro* proliferation of T cells, stimulated by mitogens or antigens, required the presence of IL 2 secreted by activated T helper cells.[6] The modifications in the response to mitogens and to allogeneic cells observed after tuftsin treatment may be the result of an altered capacity of spleen cells to release this regulatory mediator.

To investigate this point, spleen cells from tuftsin-treated mice were examined for their capacity to produce IL 2 upon *in vitro* stimulation with Con A. IL 2 was assessed in the culture supernatant after 24 hours of incubation by its discriminative property to support the proliferation of T cell blasts in the absence of an additional mitogen stimulation.

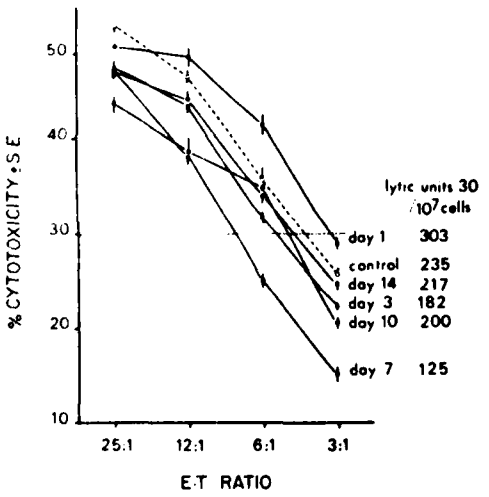

FIGURE 3. Generation of cytolytic T lymphocytes *in vitro* with spleen cells obtained at various times after a single i.v. injection of 25 μg tuftsin. Spleen cells (5×10^6/ml) from control or tuftsin-treated mice were immunized by culture with mitomycin C-treated allogeneic P815 tumor cells (6.25×10^5/ml). After 5 days, their cytotoxic activity was assessed using ^{51}Cr-labeled P815 tumor cells as targets. Results are expressed as the percentages of cytotoxicity obtained with different effector to target cell ratios (E:T) and as lytic units/10^7 immunized spleen cells, a lytic unit being defined as the number of effector cells required to lyse 30% of 1×10^4 target cells.

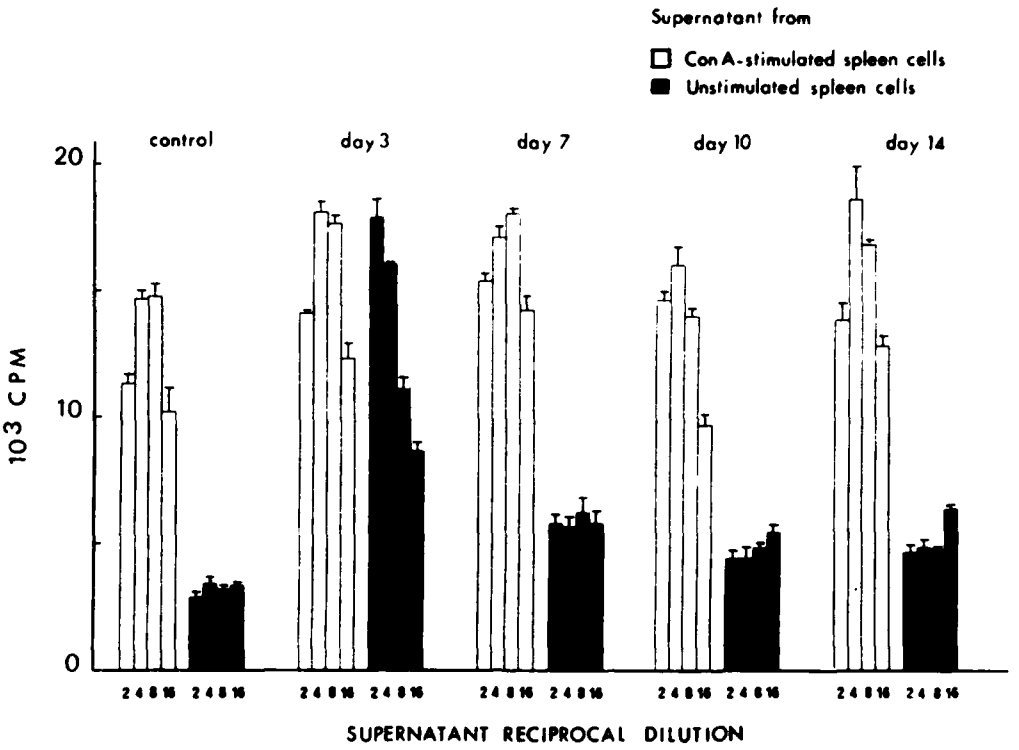

FIGURE 4. IL 2 production by spleen cells obtained at various times after a single i.v. injection of 25 μg tuftsin. IL 2 activity was assessed in the supernatant of spleen cells (2.5×10^6/ml) from control or tuftsin-treated mice, incubated for 24 hours in the presence □ or in the absence ■ of Con A (5 μg/ml), by its ability to support the proliferation of T-cell blasts obtained after stimulation of normal spleen cells with PHA for 48 hours. Results are expressed as [^{3}H]-TdR incorporation (in cpm) by 2×10^4 T-cell blasts incubated for 24 hours with various dilutions of spleen cell supernatants.

FIGURE 4 shows that spleen cells from mice given tuftsin 3, 7, or 14 days before testing produced slightly more IL 2 than normal spleen cells when stimulated *in vitro* with Con A. Moreover, spleen cells from 3-day-treated mice spontaneously produced a similar level of IL 2 as Con A-stimulated cells. This suggests that tuftsin activated *in vivo* IL 2-producing cells.

Effect of Tuftsin Administration on Phagocytosis-Induced Chemiluminescence of Peritoneal Cells

We have already reported that macrophages from tuftsin-treated mice displayed an increased capacity to ingest and to kill bacteria.[5,7] The microbicidal action of macrophages and PMN is thought to proceed in part through oxidative mechanisms.[8] Following phagocytosis, reactive species of oxygen are generated that induced light emission by the cells.[9] This chemiluminescence, which is amplified in the presence of easily oxidizable substances like luminol, can be used as an indirect assay of phagocytic and bactericidal functions of macrophages and PMN.[10]

At different times after tuftsin injection, peritoneal cells were examined for luminol-enhanced chemiluminescence upon phagocytosis of opsonized zymosan particles. In this experiment, light emission was recorded continuously for 30 minutes with a photomultiplier connected to an amperemeter.

TABLE 1. Phagocytosis-induced Chemiluminescence of Peritoneal Macrophages at Various Times after a Single Injection of 25 μg Tuftsin[a]

Route of Administration	Superoxide Dismutase (15 IU/ml)	– (Controls)	Day After Tuftsin Injection 1	3	7	10	14
			Peak of Chemiluminescence (10^{-9} A)				
i.v.	–	1.2	4.0	7.9	6.7	8.1	9.4
	+	1.2	2.1	2.8	2.0	2.3	3.5
i.p.	–	1.3	3.4	7.7	6.7	5.8	6.2
	+	1.2	2.1	3.2	3.1	3.0	3.2

[a]Chemiluminescence emitted by peritoneal cells (4×10^6/2 ml) was recorded continuously for 30 min with a photomultiplier connected to a picoamperemeter in the presence of luminol (5×10^{-6} *M*) after addition at time 0 of zymosan (1.5 mg/ml) opsonized with normal mouse serum and in the presence or in the absence of superoxide dismutase.

Results presented in TABLE 1 show that tuftsin stimulated phagocytosis-induced luminescence to a similar extent irrespective of whether it was injected i.v. or i.p. This stimulatory effect was already detectable one day after treatment and remained very important on day 14. When measurements were performed in the presence of superoxide dismutase, a 48% to 72% reduction in chemiluminescence emission was observed. This demonstrates that O_2^- but also other reactive species of oxygen were involved in the genesis of chemiluminescence.

Effect of Tuftsin Administration on Macrophage Cytostatic Activity on Tumor Cells

Bactericidal and tumoricidal activities are generally acquired simultaneously by macrophages during an activation process. Peritoneal macrophages from tuftsin-treated mice were therefore examined for their capacity to inhibit tumor cell proliferation *in vitro*. P815 mastocytoma cells were exposed for 24 hours to macrophages and allowed to incorporate [^{3}H]-TdR for the last 4 hours of incubation. In the first experiment, macrophage cytostatic activity was detected 3 days after i.v. injection of tuftsin. In the second experiment, this cytostatic activity was more important since up to a 95% reduction of thymidine incorporation was observed, but it required a lag time of 7 days to develop (FIGURE 5).

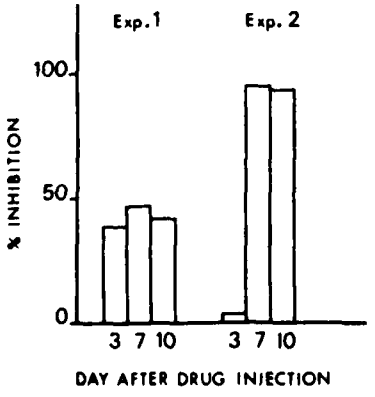

FIGURE 5. Cytostatic activity on tumor cells of resident peritoneal macrophages obtained at various times after a single i.v. injection of 25 μg tuftsin. Results are expressed as the percentage of inhibition of [^{3}H]-TdR incorporation in P815 tumor cells exposed for 24 hours to tuftsin-treated macrophages as compared to the incorporation in the presence of macrophages from control mice (macrophage to tumor cell ratio = 10:1).

Effect of Tuftsin Administration on Antibody-Dependent Cellular Cytotoxicity (ADCC)

ADCC against antibody-coated chick erythrocytes is considered to be mediated by macrophages in mice.[11] As shown in FIGURE 6, i.v. administration of tuftsin resulted in a marked increase in ADCC activity of spleen cells that was already maximal one day after the treatment. This stimulatory effect was more evident when ADCC activity was expressed at the level of the whole organ (lytic units per spleen) rather than on a cell to cell basis. This is due to the fact that tuftsin administration is followed by spleen enlargement. A 1.6- to 2.4-fold increase in the number of lytic units 50 (number of spleen cells required to lyse 50% of the target cells) per spleen was observed throughout the 14-day period of observation.

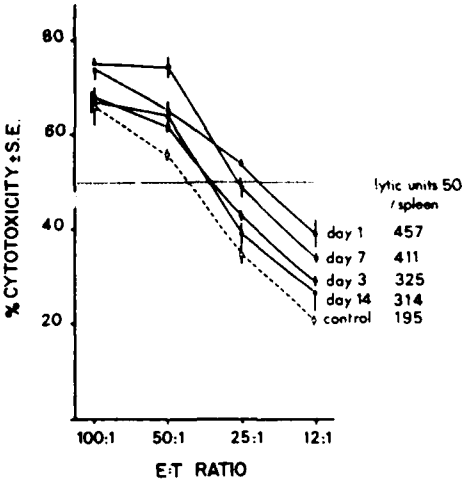

FIGURE 6. ADCC activity of spleen cells obtained at various times after a single i.v. injection of 25 μg tuftsin. Various numbers of spleen cells from control or tuftsin-treated mice were incubated for 18 hours with 1×10^4 ^{51}Cr-labeled chick erythrocytes in the presence of rabbit anti-chick erythrocyte antiserum (final dilution 1:20 000). Results are expressed as the percentages of cytotoxicity obtained for different effector to target cell ratios (E:T) and as lytic units/10^7 spleen cells, a lytic unit being defined as the number of effector cells required to lyse 50% of 1×10^4 target cells.

Effect of Tuftsin Administration on Natural Killer (NK) Cell Activity

NK cells, with macrophages and polymorphonuclear leukocytes, are involved in natural host resistance against cancer and infectious diseases.[12] As with these other effector cells, NK cells have spontaneous cytotoxic activity that can be rapidly augmented by a variety of stimuli.[13] Philipps *et al.*[14] have reported that NK cell activity is markedly increased after *in vitro* exposure to tuftsin. We therefore investigated whether natural killing could be modified after *in vivo* treatment. Results presented in FIGURE 7 show that after i.v. or i.p. administration of tuftsin, there was a slight increase in the cytotoxic activity of spleen cells against YAC-1 lymphoma cells, a very sensitive target to NK effector cells, 1 or 3 days after the treatment whereas a slight inhibitory effect may be observed later on.

The cytotoxic activity of peritoneal cells, which is very low in normal mice, was not modified after i.v. injection of tuftsin. In contrast, i.p. administration resulted in a dramatic 18-fold increase in NK activity on day 1. This stimulatory effect rapidly decreased thereafter, becoming undetectable on day 10. This decrease coincided with macrophage activation. It is therefore possible that activated macrophages released products such as prostaglandins and hydrogen peroxide that are known to inhibit NK activity.[15,16]

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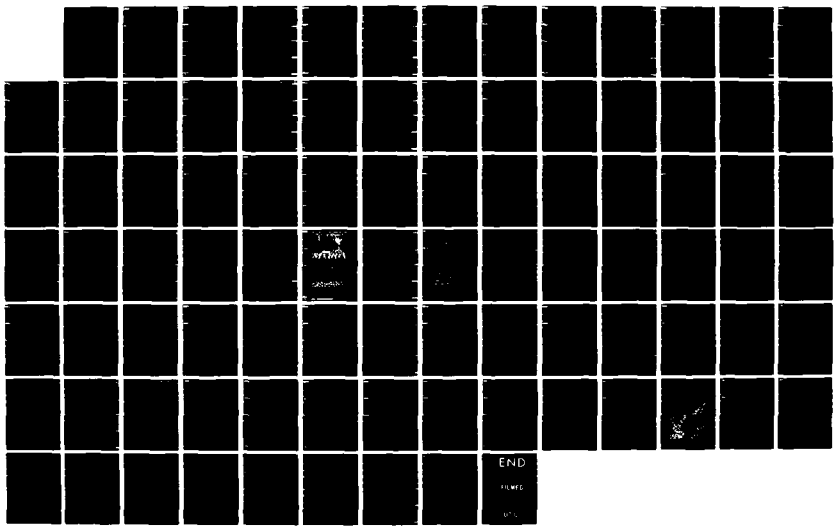

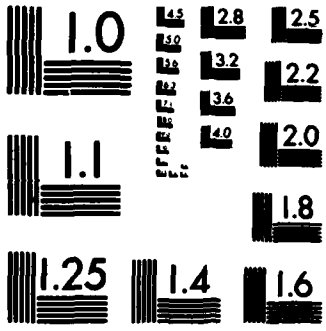

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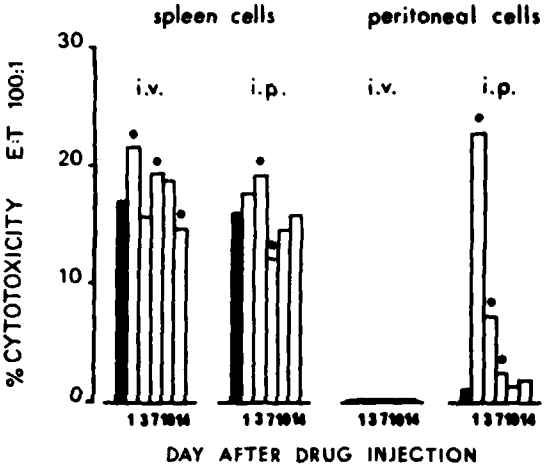

FIGURE 7. NK activity of spleen and peritoneal cells obtained at various times after a single i.v. or i.p. injection of 25 μg tuftsin. Spleen and peritoneal cells from control mice ■ and from tuftsin-treated mice □ were incubated for 4 hours with 1×10^4 ^{51}Cr-labeled YAC-1 tumor cells at an effector to target cell ratio of 100:1. * indicates that the cytotoxic activity of tuftsin-treated cells is significantly different ($p < 0.05$) from that of the control cells.

In Vitro *Immunomodulating Activity of Tuftsin*

Polymorphonuclear leukocytes (PMN) and macrophages appeared as the main, if not the only, target cells of tuftsin action taking as a basis the presence of specific receptors on these cells. In the second part of this work, we looked at modifications of their functions involved in the mechanisms of defense against tumors and infections namely, chemotactic, phagocytic, and cytotoxic activities after *in vitro* cell exposure to various concentrations of the peptide.

Effect on Chemotactic Activity of PMN

Chemotaxis is defined as directed movement of a cell towards an attractant. It is the mechanism by which the leukocytes are attracted to sites of inflammation and infection.

In the present experiments, chemotaxis was assessed by the method described initially by Bessis[17] and modified by Giroud *et al.*[18] whereby the movement of PMN towards a laser-lysed erythrocyte is followed under a phase-contrast microscope. To

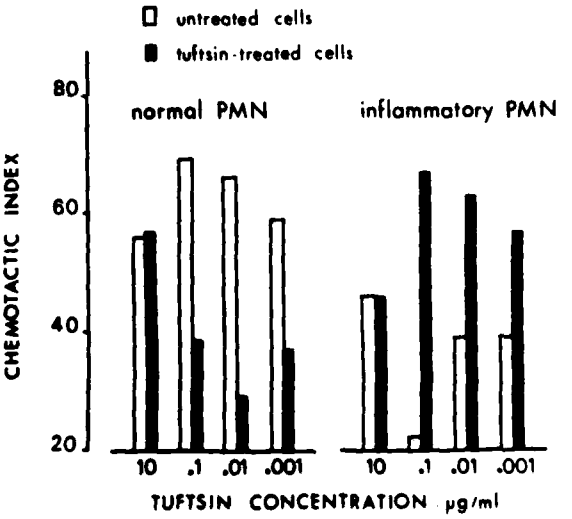

FIGURE 8. Chemotactic response of normal and inflammatory PMN after *in vitro* exposure to tuftsin. PMN were obtained from the pleural cavity of rats 4 hours after a local injection of isologous serum (normal PMN) or calcium pyrophosphate microcrystals (inflammatory PMN). Cells were incubated for 15 min with various concentrations of tuftsin and washed before observation of their chemotactic migration towards a laser-lysed erythrocyte. A chemotactic index is calculated that represents the ratio between the number of PMN on the target 10 min after erythrocyte lysis and the number of PMN in the field initially.

quantify the response, a chemotactic index was calculated that represents the ratio between the number of cells on the target red cell 10 minutes after lysis to the number of PMN in the field initially.

PMN were obtained from the pleural cavity of rats 4 hours after a local injection of either isologous serum (normal PMN) or a calcium pyrophosphate microcrystal suspension (inflammatory PMN). In both cases, the pleural exudate contained more than 90% PMN. As shown in FIGURE 8, the chemotactic response of normal PMN was markedly inhibited after preincubation for 15 minutes with 10^{-1} to 10^{-3} μg/ml of tuftsin. Untreated inflammatory PMN show a decreased chemotactic reactivity as compared to normal PMN. In this case, tuftsin pretreatment allowed a restoration to normal values of this defective response except when the cells were exposed to the highest concentration (10 μg/ml) of tuftsin, which was also ineffective on normal PMN. The specificity of the reaction was demonstrated by the lack of action of "retro tuftsin" which has the same amino acid composition as tuftsin but in a different sequence (results not shown). The cell velocity, which was determined under microci-

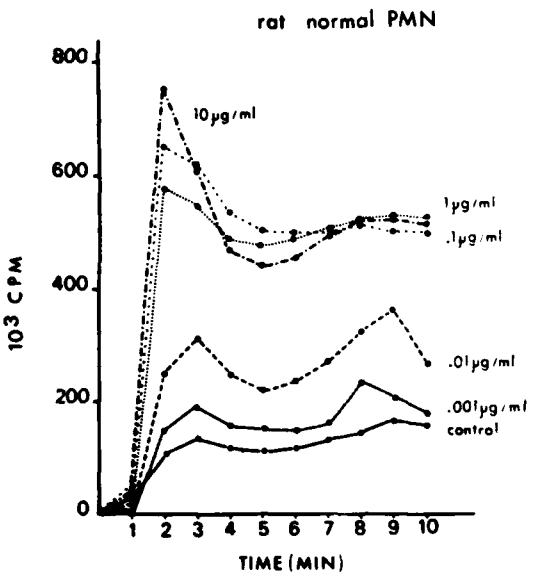

FIGURE 9. Time-course of chemiluminescence emitted by normal PMN, in response to opsonized zymosan after *in vitro* exposure to tuftsin. Cells (6×10^5/ml) were incubated for 15 min with various concentrations of tuftsin, washed and examined for light emission after addition of luminol (5×10^{-6} *M*) and zymosan (1.5 mg/ml) opsonized with normal rat serum.

nematography by the measurement of the distance traveled by individual cells over a 3-minute period, was not significantly modified by tuftsin pretreatment irrespective of whether normal or inflammatory PMN were used (results not shown). It can be noticed that Nishioka *et al.*[19] observed an increase of PMN motility in the presence of tuftsin. In our experiment, tuftsin was removed before assessment of PMN activity.

Effect of Phagocytic Activity of PMN and Macrophages

In this series of experiments, luminol-enhanced chemiluminescence emitted upon phagocytosis of opsonized zymosan particles by normal or inflammatory PMN and by resident peritoneal macrophages after 15 minutes of incubation with tuftsin was measured using a Packard Picolite luminometer.

A sharp increase in phagocytosis-induced chemiluminescence was observed when normal PMN were exposed to 10, 1, or 0.1 μg/ml of tuftsin. This stimulatory effect was less evident with further decrease in the concentration (FIG. 9). When inflamma-

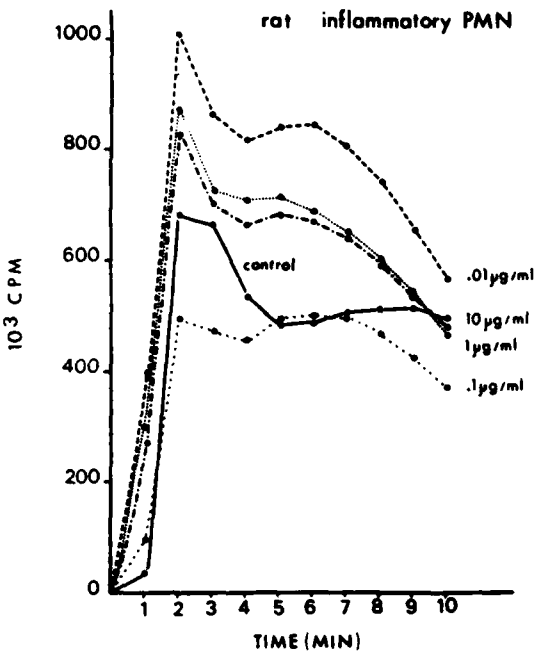

FIGURE 10. Same as in FIGURE 9, using inflammatory PMN in place of normal PMN.

tory PMN were used, it first appeared that these cells emitted more chemiluminescence than normal PMN. Tuftsin pretreatment was able to further increase this activity; the most effective concentration was 0.01 μg/ml, whereas 0.1 μg/ml was practically without effect (FIG. 10).

Normal resident peritoneal macrophages emitted much less chemiluminescence upon phagocytosis than normal PMN. A 10, 7, and 5-fold increase in this activity was observed after cell exposure, respectively, to 1, 0.01, and 0.001 μg/ml of tuftsin, whereas 10 μg and 0.1 μg/ml were quite inactive (FIG. 11).

Effect on Macrophage Cytostatic Activity

Normal resident peritoneal macrophages, after a 48-hour incubation with 1 or 0.1 μg/ml of tuftsin, inhibited by approximately 70% the proliferation of P815 mastocy-

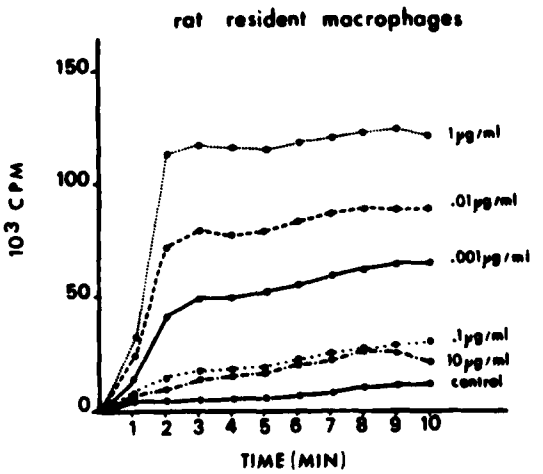

FIGURE 11. Time-course of chemiluminescence emitted by peritoneal resident macrophages upon phagocytosis of opsonized zymosan after *in vitro* exposure to tuftsin. Cells (4×10^6) were incubated for 15 min with various concentrations of tuftsin, washed and examined for light emission as described for PMN.

TABLE 2. Cytostatic Activity on Tumor Cells of Peritoneal Macrophages after *in Vitro* Exposure to Tuftsin[a]

	Normal Macrophages Incubated with:		
	Medium Alone	0.1 μg/ml Tuftsin	1 μg/ml Tuftsin
[^{3}H]-TdR incorporation by tumor cells (cpm ± SE)	7 208 ± 1 769	2 4 88 ± 535	1 671 ± 711
% Inhibition [^{3}H]-TdR incorporation	–	66%	77%

[a]Resident peritoneal macrophages (2×10^6/ml) from normal mice were incubated in the presence or in the absence of tuftsin for 48 hours before the addition of P815 mastocytoma cells (2×10^5/ml). [^{3}H]-TdR was added for 4 hours after 24 hours of contact between tumor cells and macrophage monolayers.

toma cells (TABLE 2). This observation is in agreement with those of Nishioka[20] using L1210 tumor cells as targets.

In Vivo *Immunomodulating Activity of Some Tuftsin Analogues*

From the observations of Tzehoval *et al.*,[2] it appears that the sequence Pro-Arg is essential for the stimulation of the immunogenic function of macrophages by tuftsin. Therefore, Martinez *et al.*[4] synthesized analogues differing from tuftsin by the amino acid in position 1 and (Gly^1)-tuftsin, (Met^1)-tuftsin, and (For-Met^1)-tuftsin were selected on the basis of their capacity to stimulate *in vitro* macrophage phagocytosis. On the other hand, it is now well established that activated macrophages can exert suppressive activity on various immune functions by releasing prostaglandin E_2.[21] It was therefore tempting to look at whether coupling indomethacin (Indo) with tuftsin (Indo-tuftsin) or with (*des*-Thr^1)-tuftsin [(Indo^1)-tuftsin] would increase its immunostimulating activity. These different tuftsin analogues were compared for their capacity to stimulate macrophage phagocytosis and NK activity of spleen and peritoneal cells when administered to mice in a dose of 25 μg/mouse and we looked at the influence of the route and the time of administration.

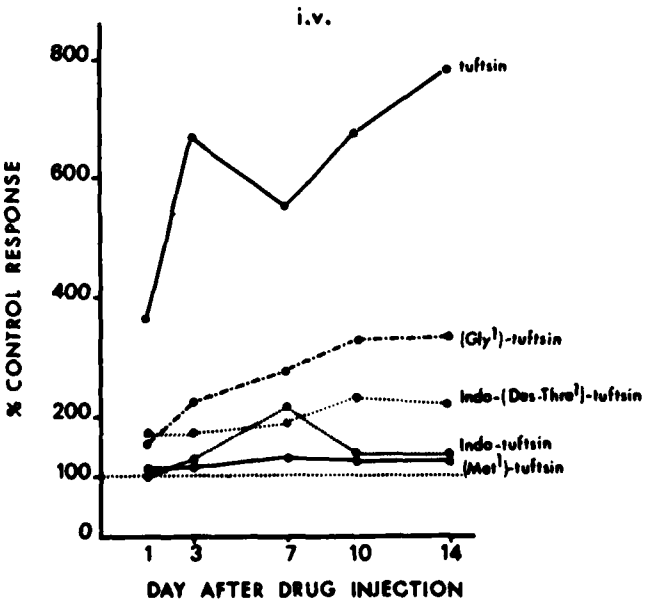

FIGURE 12. Chemiluminescence response of peritoneal resident macrophages to phagocytosis of opsonized zymosan at various times after a single i.v. injection of 25 μg tuftsin or analogues. Light emission by peritoneal cells (4×10^6) from control or treated mice was recorded continuously for 30 min after the addition of opsonized zymosan in the presence of luminol. Peak of chemiluminescence was noticed for each experimental group and results were expressed as the percentage of control chemiluminescent response.

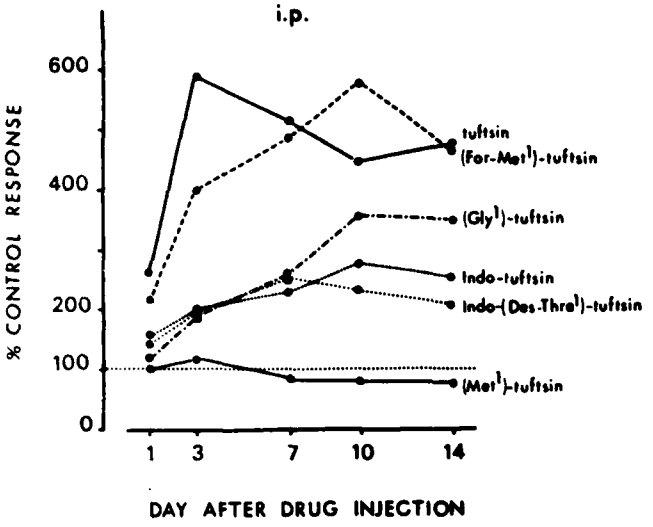

FIGURE 13. Same as in FIGURE 12, administering 25 μg tuftsin or analogues by i.p. injection.

Effect of Tuftsin Analogue Administration on Macrophage Phagocytosis

As shown in FIGURE 12, when administered i.v., tuftsin was markedly the most effective in stimulating phagocytosis-induced chemiluminescence of mouse peritoneal macrophages. (Gly¹)-tuftsin showed a moderate stimulatory activity, Indo-tuftsin and (Indo¹)-tuftsin a low one, whereas (Met¹)-tuftsin was without effect. (For-Met¹)-tuftsin was not tested in this experiment.

A quite similar picture was observed when the drugs were injected i.p. (FIG. 13). We find that the order of decreasing activity is (Gly¹)-tuftsin, Indo-tuftsin, (Indo¹)-tuftsin and (Met¹)-tuftsin, which was still inactive. In addition, (For-Met¹)-tuftsin exhibited a stimulatory activity of macrophage phagocytosis similar to tuftsin.

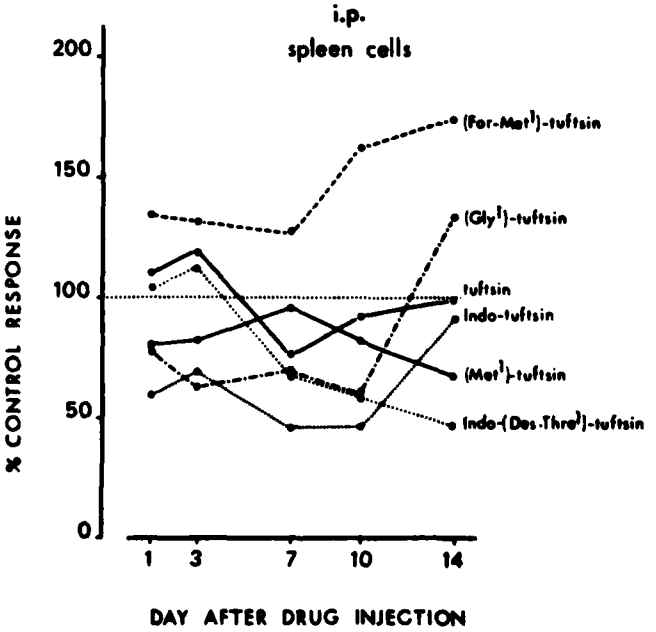

FIGURE 14. NK activity of spleen cells at various times after a single i.p. injection of 25 μg tuftsin or analogues. Cytotoxicity was measured against ^{51}Cr-labeled YAC-1 tumor cells at an effector to target cell ratio of 100:1. Results are expressed as the percentage of the cytotoxic activity of spleen cells from control mice of each experiment.

Effect of Tuftsin Analogues on NK Activity

When looking at the effect of i.p. administration of the analogues on the NK activity of spleen cells, it appears from FIGURE 14 that (For-Met[1])-tuftsin is the only analogue that stimulated this activity throughout the 14-day period of observation. Moreover, it induced the highest level of cytotoxicity that was observed 14 days after the injection. A slight increase of spleen cell cytotoxicity was also observed early (1 to 3 days) after injection of tuftsin and (Indo[1])-tuftsin and later (14 days) after (Gly[1])-tuftsin injection. Only (Indo[1])-tuftsin and (Met[1])-tuftsin exerted inhibitory effects. Concomitantly, the cytotoxicity of peritoneal cells, which did not exceed 3% in control mice, was more or less stimulated by all analogues 1 day after i.p. injection (FIG. 15). The most effective in stimulating NK activity were (Indo[1])-tuftsin, (For-Met[1])-tuftsin and tuftsin, which elevated the cytotoxicity up to 20–25%. A progressive return to control values was observed between days 3 and 10. A second peak in enhanced cytotoxicity occurred on day 14 with all analogues except after injection of (Met[1])-tuftsin and tuftsin.

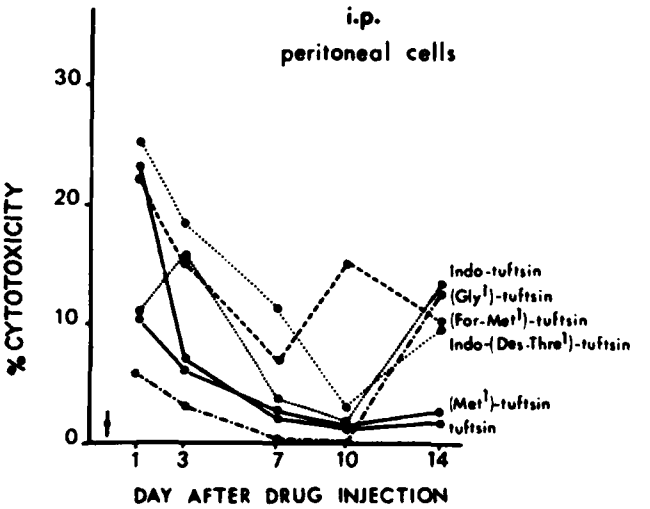

FIGURE 15. NK activity of peritoneal cells at various times after a single i.p. injection of 25 μg tuftsin or analogues. Results are expressed as the percentage of cytotoxicity against ^{51}Cr-labeled YAC-1 tumor cells for an effector to target cell ratio of 100:1. On the left is indicated the mean of the percentages of cytotoxicity ± SE of peritoneal cells from control mice of all experiments.

DISCUSSION AND CONCLUSIONS

From this work, it appears that tuftsin administered *in vivo* modulated humoral and cell-mediated immune functions. As for other immunomodulatory compounds, the dose and the time of administration are critical parameters. Tzehoval *et al.*[2] have already shown that *in vitro* stimulation of macrophage immune function by tuftsin does not occur in the presence of high concentrations of tuftsin. This finding is in agreement with our observation that antibody responses are depressed in mice given a high dose (400 μg) of tuftsin. One possible explanation is that the metabolic degradation of tuftsin resulted in the liberation of the more stable tripeptide Lys-Pro-Arg, which was shown to be an inhibitor of tuftsin activity and to compete with the tetrapeptide for cellular receptors.[22,23]

Another point that deserves to be underlined is that some of the immunomodulatory effects of tuftsin were observed at least up to 14 days after administration of this peptide. This suggests that tuftsin binding to cell receptors initiated a cascade of events

involving complex cell to cell interactions. In addition, all effects on immune functions that were observed may be the result of a primary action of tuftsin on macrophages acting as regulatory cells or as effector cells.

Our observation on the *in vitro* effect of tuftsin on PMN chemotaxis shows that according to their physiopathological state (inflammatory versus normal PMN) cells may respond in an opposite way to tuftsin. Similarly, as shown by Bruley-Rosset *et al.*,[24] age-immunodepressed mice did not respond in the same way as immunocompetent young adult mice to *in vivo* administration of tuftsin since NK activity that is depressed in aged mice was unaffected by tuftsin treatment, impaired T-cell cytotoxicity was strongly stimulated, whereas ADCC was inhibited. Only macrophages respond equally well to tuftsin and became highly cytostatic for tumor cells irrespective of the age of mice. These findings may have clinical implications since tuftsin, like other immunomodulators, will be administered to patients with impaired immune functions and even with organ dysfunctions which may alter the pharmacokinetics of the compound.

From our studies on analogues, it appears that threonyl in position 1 is not essential for the *in vivo* stimulating activity of macrophage phagocytosis and natural killing. Among the analogues tested (For-Met^1)-tuftsin was the most effective one, whereas (Met^1)-tuftsin was practically inactive. Therefore, the formyl group seems to be important, likely by conferring some resistance to degradation. Our observations, in addition to the ones of Fridkin *et al.*[22] showing that, *in vitro*, the stimulatory effect of tuftsin on nitroblue tetrazolium reduction by PMN is very much more dependent upon the integrity of the Thr^1 residue than is the stimulation of PMN phagocytosis, suggest that structure requirement for activity may vary according to the cell function affected.

From the data obtained by others (see ref. 23 for review) and us with *in vitro* and *in vivo* experiments in various physiological and pathological conditions, it appears that tuftsin could be considered an important therapeutic agent for stimulating the defense mechanisms against infections and neoplasia.

ACKNOWLEDGMENTS

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Tuftsin-induced Enhancement of Murine and Human Natural Cell-mediated Cytotoxicity[a]

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INTRODUCTION

The importance of cell-mediated immune responses in the normal host's control of bacterial and viral infections, foreign antigens, and the rejection of tumors has been well documented now for many years. Recently, the phenomenon of natural cell-mediated cytotoxicity against tumor cells without apparent presensitization has gained recognition as an important function component of the normal immune armamentarium against neoplastic growth. Natural cell-mediated cytotoxicity can be mediated by a variety of effector cells, including macrophages, monocytes, granulocytes, mast cells, and NK (natural killer) cells. Since natural cell-mediated mechanisms may function to retard neoplastic growth, substantial research has been devoted to identifying the factors and agents that modulate the immune system. Tuftsin is an endogenous tetrapeptide, which has recently been shown to possess immunoadjuvant properties, including antitumor potential.[1,2] In view of the evidence indicating the antineoplastic characteristics of tuftsin, we have undertaken a series of studies to determine if tuftsin directly affects natural cell-mediated cytotoxicity against tumors.

MATERIALS AND METHODS

Mice

Three- to four-month-old male CBA/J mice were obtained from Dr. Geoffery Haughton, University of North Carolina, Chapel Hill, N.C. and C57BL/10 ScSn mice were raised in our laboratory from breeding stocks also supplied by Dr. Haughton.

Culture Medium

The medium used for tumor cell cultures and all cytotoxicity assays was RPMI 1640 (Grand Island Biological Co., Grand Island, NY) supplemented with 10%

[a]This research was supported in part by grant CA 27330 from NCI, U.S. Public Health Service.

heat-inactivated fetal calf serum (FSC), 20 m*M* Hepes, 0.03% L-glutamine, and antibiotics.

Tumor Cells

Yac-1, a T-cell lymphoma of A/Sn origin was used as a murine NK-sensitive target and was kindly supplied by Drs. L. L. Lanier and N. Warner, Becton-Dickinson, Monoclonal Antibody Center, Mountain View, CA. K562, a human erythromyeloid leukemia, was used as target in the human system. The tumor cells used in this paper were maintained as continuous cultures in our laboratory.

Tuftsin

Synthetic tuftsin was obtained from Takeda Chemical Co., Osaka, Japan, and analyzed for purity by high-performance liquid chromatography. Purified tuftsin was stored in crystallized form at 4°C and was diluted to appropriate concentration in either SCM or Hanks balanced salt solution (HBSS).

Cytotoxicity Assay

Tumor cells were labeled with 100 μCi of radioactive sodium chromate (^{51}Cr, specific activity of 500 mCi/mg, New England Nuclear, Boston, MA) according to the methods originally described by Kiessling *et al.*[3] and performed by us.[4] After treatment of effector cells with tuftsin or control medium, effector cells (at various concentrations) and 1×10^4 target cells were added to each well in total volume of 0.2 ml/well. The plates were then agitated and incubated for 4, 6, 18, or 30 hours at 37°C. After incubation, 0.05 ml of cold medium was added to each well and the plates centrifuged. A 0.1 ml aliquot was then removed from each well and counted in a Gamma Spectrometer. Percent cytotoxicity was computed according to the following formula:

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental C.P.M.-Spontaneous C.P.M.}}{\text{Maximum C.P.M.-Spontaneous C.P.M.}} \times 100$$

Spontaneous release of chromium was determined by incubating target cells in medium alone; it ranged from 6 to 8% for 4-hr assays, 20 to 30% for 18-hr assays, and 30 to 40% for 30-hr assays. Maximum release of chromium was determined by incubating target cells in 10% Triton X-100 and ranged from 80 to 95% of total incorporated radioactivity. All assays were performed 4–6 times and results are expressed as mean percent cytotoxicity standard error of the means. Results were evaluated statistically with Student's t-test. Probability values $p < 0.05$ were considered statistically significant.

Effector Cell Preparations

Mice were sacrificed by cervical dislocation. Spleen and bone marrow suspension were prepared in Hanks balanced salt solution (HBSS). Red cells were lysed with distilled water and debris removed by passage through a 200-mesh stainless steel screen. Single cell suspensions were then prepared in SCM at appropriate cell

concentrations. In some experiments, murine splenic suspensions were fractionated as follows: (1) Macrophages were removed and collected by plating on plastic petri dishes; (2) adherent and immunoglobulin-bearing cells were removed by characteristic adherence to nylon wool; and (3) Nylon wool nonadherent cells were then depleted of T cells by treatment with monoclonal anti-Thy 1.2 antibodies and complement.[5] Characterization of murine effector suspensions was performed as previously described.[4]

For human studies, heparinized peripheral blood was donated by healthy volunteers. Leukocyte-enriched suspensions were obtained by dextran sedimentation ($1 \times g$) at 37°C for 1 hr. Mononuclear leukocytes were isolated at the interface of ficoll-isopaque gradients, while purified granulocytes were recovered from the pellets. The purity of the granulocyte suspension was determined morphologically. Monocytes were removed and collected from the mononuclear leukocyte suspensions by two sequential plastic adherences at 37°C. The monocyte suspensions were 85–90% pure as determined by nonspecific esterase staining[6] and indirect immunofluorescence using the monoclonal anti-human monocyte antibody M.2 (Bethesda Research Laboratories, Gaithersburg, MD). Any residual adherent cells and immunoglobulin-bearing B cells were then removed from the plastic nonadherent suspensions by passage through nylon wool.[7] Human NK cells were purified on discontinous gradients of Percoll by a procedure similar to that recently reported by Timonen *et al.*[8] The purity of the NK-cell suspensions was 85–90% as determined by morphology and indirect immunofluorescence using the NK-cell reactive monoclonal antibody Leu-7 (Becton-Dickinson, CA).[9]

In Vitro *Treatment of Effector Cells with Tuftsin*

One-tenth milliliter of effector cells (5×10^6 cells/ml) was plated in triplicate into wells of flat-bottomed microtiter plates (Falcon Plastics), to which was added 0.05 ml of various concentrations of tuftsin. Plates were then incubated for 1 hr at 37°C before the addition of target cells.

In Vitro *Pretreatment of Effector Cells with Tuftsin*

One milliliter of effector cells (5×10^6 cells/ml) was incubated with 0.5 ml of various concentrations of tuftsin in 6 × 10 mm glass test tubes at 37°C for 0.5, 1, 3, or 6 hr. After pretreatment incubations, the cells were washed thoroughly in SCM.

In Vivo *Injection of Tuftsin into C57BL/10 Mice*

C57BL/10 mice were injected intravenously via the lateral tail vein with various concentrations of tuftsin dissolved in 0.5 ml of HBSS. Control mice were injected in with HBSS alone. Various times after tuftsin injection, the mice were sacrificed and splenic and bone marrow suspension were monitored for natural cell-mediated cytotoxicity.

RESULTS

In Vitro *Effects of Tuftsin on Murine Natural Cell-mediated Cytotoxicity*

TABLE 1 summarizes the results of the *in vitro* effects of tuftsin on murine natural cell-mediated cytotoxicity. CBA/J mice were employed on the initial studies. Three

TABLE 1. *In Vitro* Effects of Tuftsin on Murine Natural Cell-mediated Cytotoxicity[a]

Effector Cells[b]	Tuftsin (μg/ml)[c]						
	0	0.01	.1	1	10	100	1000
Splenic macrophages	5 ± 1.5	7 ± 1.0	10 ± 1.5(*)	16 ± 2.0(*)	17 ± 1.3(*)	12 ± 2.0(*)	4 ± 2.0
Peripheral blood granulocytes	1 ± 1.5	7 ± 2.0(*)	9 ± 1.5(*)	12 ± 2.0(*)	13 ± 3.0(*)	12 ± 2.0(*)	5 ± 1.5(*)
Splenic NK cells	35 ± 2.0	31 ± 3.0	40 ± 4.0	50 ± 3.0(*)	54 ± 2.0(*)	71 ± 4.0(*)	28 ± 4.0

[a]Results expressed as % cytotoxicity ±SE. Cytotoxicity assay was a standard 18-hr ^{51}Cr-release assay using Yac-1 as a tumor target and an effector-to-target ratio of 50:1. SE, standard error of the mean. (*), $p < 0.05$.

[b]Effector cells obtained from CBA/J mice. Macrophages isolated by plastic adherence were 85–90% nonspecific esterase positive and phagocytic. Granulocytes were collected by Ficoll-Hypague centrifugation. Enriched NK-cell suspensions were obtained by depleting nylon wool nonadherent suspensions of T cells.

[c]Various concentrations of tuftsin were combined with effector cells for 1 hour before addition of tumor target cells.

purified/enriched effector cell populations were investigated: splenic macrophages, NK cells and peripheral blood granulocytes. Treatment of all three effector cell populations with various concentrations of tuftsin induced a substantial dose-dependent enhancement of natural cell-mediated cytotoxicity against the T-cell lymphoma Yac-1. The lowest concentration of tuftsin to induce significant stimulation of cytotoxicity, however, was different for each effector population. A tuftsin concentration of 0.1 μg/ml was required to enhance macrophage-mediated cytotoxicity 2-fold (from 5% to 10%). Granulocyte-mediated cytotoxicity, however, required only a tuftsin concentration of 0.01 μg/ml to stimulate cytotoxicity 7-fold (from 1% to 7%). Splenic NK cells required a substantially higher concentration of tuftsin (1 μg/ml) to significantly enhance cytotoxicity above control levels. A maximum stimulation of natural cell-mediated cytotoxicity was observed at a tuftsin concentration of 10 μg/ml for macrophages and granulocytes, and 100 μg/ml for NK cells. The higher concentration of tuftsin employed in this study (1000 μg/ml) had no effect on monocyte or NK cell-mediated cytotoxicity, however, granulocyte-mediated cytotoxicity continued to demonstrate tuftsin-induced enhancement of cytotoxicity.

In Vitro *Effects of Tuftsin on Murine Natural Cell-mediated Cytotoxicity: Varied Assay Time*

In these experiments, C57BL/10 effector cell populations were treated with optimal concentrations of tuftsin, combined with Cr-labeled Yac-1 cells and incubated for 4, 18, or 30 hr at 37°C. TABLE 2 summarizes the results of these experiments. When the cytotoxicity assay was terminated at 4 hr, only splenic NK cells showed significant cytotoxicity against Yac-1. Likewise, only NK cells showed significant tuftsin-induced enhancement of cytotoxicity, approximately 2-fold during a 4-hr assay. As previously described, when the assay was extended to 18 hr, all three effector populations showed substantial tuftsin-induced stimulation of natural cell-mediated cytotoxicity. In C57BL/10 mice, tuftsin-induced enhancement of cytotoxicity was approximately 3-fold for macrophages, 7.5-fold for granulocytes, and 2.5-fold for NK cells. When the cytotoxicity assays were extended to 30 hr, all three effector populations showed cytotoxicity levels significantly greater than those observed at 18 hr. Macrophages demonstrated an overall tuftsin-induced enhancement of 4.5-fold over unstimulated

TABLE 2. *In Vitro* Effects of Tuftsin on Murine Natural Cell-mediated Cytotoxicity[a]

Effector Cells[b]	Tuftsin[c] (μg/ml)	Assay Time (hours) 4	18	30
Splenic macrophages	0	1 ± 1.5	6 ± 2.0	8 ± 2.5
	10	2 ± 1.5	18 ± 2.0(*)	35 ± 4.5(*)
Peripheral blood granulocytes	0	1.5 ± 1.0	2 ± 1.5	3 ± 2.0
	10	<1	15 ± 2.5(*)	26 ± 3.3(*)
Splenic NK cells	0	8 ± 1.5	12 ± 2.3	15 ± 2.5
	50	19 ± 2.0(*)	29 ± 3.5(*)	55 ± 4.0(*)

[a]Results expressed as % cytotoxicity ± SE. Cytotoxicity was assessed using ^{51}Cr-release assay with Yac-1 as a tumor target and effector-to-target ratio of 50:1. Assays were terminated at 4, 18, and 30 hr. (*), $p < 0.05$.

[b]Effector cells prepared as described in MATERIALS AND METHODS from C57BL/10 mice.

[c]Various concentrations of tuftsin were incubated with effector cells for 1 hour before the addition of target cells.

TABLE 3. *In Vitro* Effects of Pretreatment with Tuftsin on Murine Natural Cell-mediated Cytotoxicity[a]

Effector Cells[b]	Tuftsin[c] (μg/ml)	Pretreatment Times (hours)[d] 0.5	1	3	6
Splenic macrophage	0	3 ± 1.5	5 ± 2.5	3 ± 2.0	4 ± 2.0
	10	10 ± 2.0(*)	19 ± 3.5	25 ± 3.0(*)	22 ± 2.0(*)
Peripheral blood granulocyte	0	1 ± 1.5	3 ± 2.0	2 ± 1.5	2 ± 2.0
	10	9 ± 2.0(*)	14 ± 2.0(*)	18 + 3.0(*)	20 ± 2.5(*)
Splenic NK cell	0	33 ± 3.0	30 ± 1.5	24 ± 2.0	16 ± 3.0
	100	31 ± 2.5	62 ± 3.5(*)	59 ± 2.5(*)	61 ± 3.0(*)

[a]Cytotoxicity was assessed using an 18-hour ^{51}Cr-release assay and Yac-1 tumor cells. An effector-to-target ratio of 50:1 was employed. Results are expressed as percent cytotoxicity ± standard error of the means: (*), $p < 0.05$.

[b]Effector cells prepared as described in MATERIALS AND METHODS from CBA/J mice.

[c]Optimal concentrations of tuftsin were employed for each effector cell population.

[d]Effector cells were combined with tuftsin for various times as previously described then washed to remove the tuftsin.

controls and approximately 2-fold greater cytotoxicity over stimulated macrophages at 18 hr. Granulocytes displayed a tuftsin-enhanced cytotoxicity of approximately 8-fold greater than control levels and 1.7-fold greater than stimulated granulocytes at 18 hr. NK cells, unexpectedly, also demonstrated a tuftsin-induced enhancement of cytotoxicity approximately 3.5-fold greater than unstimulated controls and 1.5-fold greater than the tuftsin-enhanced cytotoxicity of NK cells observed in an 18-hr assay.

In Vitro *Effects of Varied Pretreatment Times with Tuftsin on Murine Natural Cell-mediated Cytotoxicity*

CBA effector populations were pretreated with either 10 μg/ml or 100 μg/ml of tuftsin (depending upon the effector cells) for 0.5 hr, 1 hr, 3 hr, or 6 hr and then thoroughly washed before being assayed for natural cell-mediated cytotoxicity. Splenic macrophages and peripheral blood granulocytes showed significant tuftsin-induced enhancement of cytotoxicity when pretreated with tuftsin for only 30 min (TABLE 3). Preliminary studies (data not shown) have indicated that these effector populations require at least 10–15 min of tuftsin pretreatment for significant enhancement of cytotoxicity. After 1 hr of tuftsin pretreatment, monocytes and granulocytes showed maximum levels of enhanced cytotoxicity that was slightly increased by further pretreatment to 3 or 6 hours. Splenic NK cells, however, required at least 1 hr pretreatment with tuftsin to cause significant enhancement of cytotoxicity. After 1 hr of incubation, untreated NK cells showed a gradual decline in cytotoxic activity. Tuftsin-treated NK cells, however, maintained their enhanced cytotoxic levels. One hour pretreatment of NK cells with 100 μg/ml tuftsin was adequate to induce maximum stimulation of cytotoxicity. Extending the pretreatment time to 3 or 6 hr did not increase the cytotoxicity beyond that observed after a 1-hr treatment.

In Vivo *Effects of Tuftsin on Murine Splenic and Bone Marrow Natural Cell-mediated Cytotoxicity*

In these experiments, C57Bl/10 mice were injected intravenously with various concentrations of tuftsin in 0.5 ml HBSS or of HBSS alone and their splenic and bone

marrow natural cell-mediated cytotoxicity assessed after 12, 24, 120, 216, or 264 hr (TABLE 4). At all tuftsin concentrations used in these experiments, significant enhancement of splenic cytotoxicity was observed 12 hr after i.v. injection. A tuftsin concentration of 100 μg induced the highest level of cytotoxicity 12 hr postinjection. At 24 hr, postinjection, all tuftsin concentrations continued to maintain enhanced levels of cytotoxicity; however, maximum levels of cytotoxicity were observed in those animals receiving 50 μg of tuftsin. By 120 hr, postinjection, all tuftsin-injected animals, except the 50 μg animals, showed cytotoxicity levels within the range of control values. Animals receiving 50 μg of tuftsin, however, continued to maintain significantly elevated levels of splenic natural cell-mediated cytotoxicity. By 216 hr, splenic natural cell-mediated cytotoxicity of all tuftsin-injected animals returned to normal control levels. When tuftsin-injected animals were assayed at 264 hr postinjection, animals that had received 50 μg or 100 μg of tuftsin showed a secondary peak of enhanced

TABLE 4. *In Vivo* Effects of Tuftsin on Murine Splenic and Bone Marrow Natural Cell-Mediated Cytotoxicity[a]

Effector Cells[b]	Tuftsin[c] (μg/0.5 ml)	Time Postinjection (hours)[d] 12	24	120	216	264
Splenic Cells	0	9 ± 2.0	10 ± 1.0	11 ± 2.0	9 ± 2.0	10 ± 2.0
	1	15 ± 2.0(*)	15 ± 1.5(*)	10 ± 3.0	7 ± 2.0	10 ± 3.0
	10	20 ± 1.9(*)	21 ± 2.0(*)	8 ± 2.0	10 ± 1.5	10 ± 1.5
	50	22 ± 3.0(*)	30 ± 2.0(*)	19 ± 1.5(*)	13 ± 2.0	28 ± 3.0(*)
	100	26 ± 3.0(*)	20 ± 3.5(*)	11 ± 1.3	12 ± 2.0	34 ± 3.0(*)
Bone marrow cells	0	2 ± 1.5	2 ± 1.0	15 ± 1.0	1.5 ± 1.0	1.7 ± 1.0
	1	3 ± 2.0	4 ± 1.5	2 ± 1.0	2 ± .5	1.0 ± .5
	10	15 ± 1.5(*)	15 ± 2.0(*)	5 ± 2.0	3 ± 1.5	2 ± 1.5
	50	18 ± 2.0(*)	16 ± 2.0(*)	12 ± 1.0(*)	8 ± 2.0(*)	1 ± 1.0
	100	12 ± 1.5(*)	10 ± 1.5(*)	2.0 ± 1.5	4 ± 1.5(*)	9.5 ± 2.0(*)

[a]Cytotoxicity was assessed in an 18-hr ^{51}Cr-release assay using Yac-1 as a target and an effector-to-target ratio of 50:1. Results are expressed as percent cytotoxicity ± standard error of the means. (*) $p < 0.05$.

[b]Unfractionated spleen and bone marrow suspensions C57BL/10 mice were used as effectors and were prepared as described in MATERIALS AND METHODS.

[c]Various concentrations of tuftsin in 0.5 ml of HBSS or 0.5 ml of HBSS alone were injected intravenously via the lateral tail vein.

[d]At 12, 24, 120, 216, and 264 hours after tuftsin or control injections, mice were sacrificed and splenic and bone marrow natural cell-mediated cytoxicity assayed.

cytotoxicity. This secondary stimulation of natural cell-mediated cytotoxicity declined to normal levels by 288 hr and remained within normal values thereafter (through 480 hr).

Bone marrow suspensions were also monitored for natural cell-mediated cytotoxicity at various times after a single i.v. injection of tuftsin (1–100 μg/animal). Unlike the splenic suspensions, enhanced bone marrow cytotoxicity was not observed in anmials that received 1 μg. Tuftsin injections of 10, 50, or 100 μg, however, induced a pronounced stimulation of bone marrow natural cell-mediated cytoxicity between 12–24 hr postinjection. By 120 hr, only animals that received 50 μg of tuftsin showed enhanced bone marrow cytotoxic activity. At 216 hr, the bone marrow, natural cytotoxicity of animals injected with 50 μg of tuftsin continued to maintain lower but enhanced levels of cytotoxicity, while bone marrow cells from animals receiving 100 μg

of tuftsin showed a small but significant secondary peak of enhanced activity. By 264 hr, the bone marrow, natural cell-mediated cytotoxicity of animals injected with 50 μg of tuftsin had returned to normal unstimulated levels, while the animals that had received 100 μg of tuftsin showed a maximum secondary peak of enhanced cytotoxicity. As was observed with the splenic suspensions, the secondary enhancement of bone marrow cyctotoxic activity in animals injected with 100 μg of tuftsin declined to unstimulated levels by 288 hr and remained at normal unstimulated levels through 480 hr.

In Vitro *Effects of Tuftsin on Human Natural Cell-mediated Cytotoxicity*

In these studies, purified human peripheral blood monocytes, granulocytes, and NK cells were treated *in vitro* with various concentrations of tuftsin and then assayed for natural cell-mediated cytotoxicity against the human erythromyeloid leukemia cell line K562. The purity of each effector cell population was determined by population-specific markers. Monocytes were defined as nonspecific esterase positive and stained with the monocyte-specific monoclonal antibody M.2. Granulocytes were enumerated by polymorphonuclear morphology and chloroacetate esterase staining. NK cells were characterized by large granular lymphocyte morphology and positive reactivity with the monoclonal antibody Leu-7. TABLE 5 summaries the results of these experiments. Treatment of all three effector cell populations with tuftsin induced a substantial dose-dependent enhancement of natural cell-mediated cytotoxicity against K562. A tuftsin concentration of 0.1 μg/ml was required to enhance monocyte and granulocyte-mediated cytotoxicity approximately 2-fold over unstimulated controls. Maximum monocyte and granulocyte-mediated cytotoxicity was observed at a tuftsin concentration of 10 μg/ml, while 1000 μg/ml appeared to have no effect on the cytotoxicity mediated by these effector cells. Purified human NK cell required a higher concentration of tuftsin (1 μg/ml) to enhance cytotoxicity above unstimulated control values. Maximum stimulation of NK cell-mediated cytotoxicity was also observed at a tuftsin concentration of 10 μg/ml; however, unlike monocyte- and granulocyte-mediated cytotoxicity, a tuftsin concentration of 1000 μg/ml induced significant enhancement of cytotoxicity.

DISCUSSION

In recent years, substantial research evidence has accumulated supporting the belief that natural cell-mediated cytotoxicity is an important antitumor effector mechanism. Since elevated natural cell-mediated cytotoxicity may function to retard neoplastic growth, substantial interest has been generated in delineating the factors and agents that modulate the levels of endogenous natural antitumor cytotoxicity. Human and murine natural, cell-mediated cytotoxicity has been shown to be enhanced by a variety agents including interferons, interleukin-2, several bacterial immunoadjuvants, viruses, plant lectins, and leukocytic pyrogen.[10-12]

In the present study, we present evidence for the enhancement of murine and human natural, cell-mediated cytotoxicity against tumor cells by the chemically defined tetrapeptide, tuftsin. Although in the present study, we employed synthetic tuftsin, tuftsin is believed to be an endogenous physiological tetrapeptide derived by enzymatic cleavage from the CH 2 domain (F_c region) of immunoglobulin G.[13,14] We have found that *in vitro* treatment of murine and human monocyte/macrophages,

TABLE 5. *In Vitro* Effects of Tuftsin on Human Natural Cell-mediated Cytotoxicity[a]

Effector Cells[b]	Tuftsin (μg/ml)[c]						
	0	0.01	.1	1	10	100	1000
Peripheral blood monocytes	4 ± 2.0	3 ± 2.5	8 ± 2.0(*)	16 ± 2.0(*)	18 ± 3.0(*)	14 ± 3.0(*)	6 ± 3.0
Peripheral blood granulocytes	7 ± 2.0	12 ± 3.0	14 ± 4.0(*)	21 ± 3.0(*)	24 ± 2.0(*)	20 ± 3.0(*)	10 ± 3.0
Peripheral blood NK cells	42 ± 3.0	47 ± 4.0	50 ± 4.0	80 ± 3.5(*)	90 ± 5.0(*)	78 ± 4.0(*)	60 ± 3.0(*)

[a]Cytotoxicity was assessed in an 18-hr ^{51}Cr-release assay for monocyte- and granulocyte-mediated cytotoxicity and a 6-hr assay for purified NK-cell-mediated cytotoxicity. K562 was used as a tumor target in all assays. An effector-to-target ratio of 50:1 was employed for monocyte and granulocyte effectors and E:T ratio of 3:1 was used for NK-cell effectors. Results are expressed as mean percent cytotoxicity ± standard error of means. (*) statistically significant, $p < 0.05$.

[b]Effector cells prepared as described in MATERIALS AND METHODS. Each effector cell population was approximately 90% pure as defined by the various population specific markers described in MATERIALS AND METHODS.

[c]Various concentrations of tuftsin were combined with effector cells for 1 hour before the addition of tumor target cells.

granulocytes, and NK cells with synthetic tuftsin induced a pronounced enhancement of natural, cell-mediated cytotoxicity. The magnitude of the enhancement was directly dependent upon the concentration of tuftsin employed. The minimum concentration of tuftsin required to induce significant enhancement of cytotoxicity was somewhat variable between the different effector populations: murine and human monocytes/macrophages required 0.1 μg/ml of tuftsin; murine granulocytes required less tuftsin, 0.01 μg/ml, while human granulocytes needed 0.1 μg/ml of tuftsin; and murine and human NK cells required substantially more tuftsin (1 μg/ml) than the other effector populations. Maximum stimulation of cytotoxicity by the various effector populations, however, was observed at 10 μg/ml for all effector populations, except murine NK cells where maximum enhancement was seen at 100 μg/ml of tuftsin. Studies investigating the specific binding of tuftsin to human granulocytes and murine macrophages have indicated that receptor saturation on these cells was obtained at substantially lower concentrations of tuftsin than was employed in this study.[15,16] The reasons for these discrepancies are at present unknown. However, the activation of cytotoxic mechanisms by tuftsin in these effector cell populations may represent a more complex interaction than a simple binding of tuftsin to its receptor. We have also observed that high concentrations of tuftsin (1000 μg/ml) were either ineffective or only slightly stimulatory for natural cytotoxicity mediated by all effector populations from both mice and humans. The mechanism of this decrease in cytotoxicity enhancement with higher tuftsin concentrations is at present unknown. This type of dose-dependent response, however, is not an uncommon observation with other biological response modifiers.[17,18] It has recently been reported that the tripeptide, L-Lys-L-Pro-L-Arg is strongly inhibitory to the actions of tuftsin.[19] It is possible that high concentrations of tuftsin *in vitro* may exaggerate the production of inhibitory peptides that could prevent or counteract the stimulatory mechanisms.

Since macrophages and granulocytes tend to require longer assay times to manifest cytotoxicity than NK cells, *in vitro* tuftsin-treated murine effector cell populations were assayed at various time periods to investigate whether varying the duration of the assay would delineate any differential effects of tuftsin on these three effector populations. When assays were terminated at 4 hr, only splenic NK cells showed significant cytotoxicity that was substantially augmented by 50 μg/ml of tuftsin. These results clearly indicate that splenic NK cell activity is stimulated by *in vitro* tuftsin treatment. Since monocyte/macrophages and granulocytes require longer *in vitro* assays to manifest detectable cytotoxicity than NK cells, the increased cytotoxic activity of these tuftsin-treated cells at 30 hr is consistent with what has been previously reported for natural cytotoxicity mediated by activated monocytes/macrophages and granulocytes.[20,22] NK cells, however, have been reported to be relatively labile at 37°C[11] and thus maximum cytolysis is generally recorded in an 18-hr assay. The results of this investigation indicate that tuftsin-treated splenic NK cells were unexpectedly more cytotoxic in a 30-hr assay than an 18-hr assay. The mechanisms of this tuftsin-maintained, NK-cell cytolytic potential beyond 18 hr are presently unknown. However, our laboratory has recently determined that tuftsin has both mitogenic and colony-stimulating activity for splenic suspensions.[23] Recently, the biological response modifier interferon, which has strong NK cell augmentation potential, has been shown to not only stimulate NK cells but also to recruit pre-NK cells and activate them into cytotoxic effectors.[24,25] It is thus possible that the tuftsin-induced maintenance of NK-cell cytolytic function beyond 18 hr is the result of tuftsin acting upon noncytotoxic pre-NK cells. It has recently been determined in the mouse that another lymphoid effector cell type, the NC cell (natural cytotoxic cell), has *in vitro* antitumor capabilities.[26] NC cells share many characteristics with NK cells and at present can not be isolated by physical separation techniques from NK cells.[26]

NC cells, unlike NK cells, do not show the *in vitro* lability when incubated for extended periods of time at 37°C. It is thus possible that tuftsin may also enhance NC cell-mediated cytotoxicity, thus increasing the cytotoxic potential of NK cell-enriched suspension beyond 18 hr. Investigations are presently underway to clarify these questions.

In the initial *in vitro* studies, tuftsin was present throughout the duration of the assays. In the subsequent series of experiments, effector cells were allowed to incubate with optimal concentrations of tuftsin for varied periods of time at 37°C before being thoroughly washed to remove excess tuftsin (TABLE 3). The results of these studies indicated that macrophage- and granulocyte-mediated cytotoxicity was significantly enhanced within a shorter tuftsin pretreatment time than NK cells. Maximum tuftsin-induced enhancement of cytotoxicity for all effector populations, however, was approximately 1 hr. At present, we are uncertain of the mechanisms through which tuftsin specifically augments macrophage, granulocyte, and NK cell-mediated cytotoxicity. Since macrophages and granulocytes are stimulated more rapidly than NK cells, it is possible that tuftsin activates different cytotoxic mechanisms in each effector population. Recent studies have shown that the augmentation of NK-cell activity by interferon initiates protein synthesis.[27] We are presently undertaking a series of experiments to determine whether DNA, RNA, and protein synthesis are required to manifest the tuftsin-induced enhancement of natural cell-mediated cytotoxicity.

In view of the evidence indicating the potential of tuftsin to function *in vitro* as an immunoadjuvant for various effector populations and *in vivo* as an antineoplastic agent,[2] we investigated the *in vivo* effects of tuftsin on natural cell-mediated cytotoxicity. A single injection of tuftsin given intravenously into C57BL/10 mice produced a strong enhancement of splenic and bone marrow natural, cell-mediated cytotoxicity with a time kinetics similar to that observed for other biological response modifiers. Tuftsin augmentation was detectable in the spleen and bone marrow by 12 hr postinjection, reached peak activity by 1–2 days, and declined to normal baseline levels on day 5–7. Unlike other biological response modifiers, however, animals that received higher concentrations of tuftsin (50–100 μg) demonstrated a secondary peak of augmented cytotoxicity at approximately day 11, which declined to and remained at baseline levels by day 12–13. The *in vivo* mechanisms of tuftsin-induced stimulation of natural cell-mediated cytotoxicity are presently unknown. Preliminary effector fractionation studies (data not shown) has indicated that the augmented cell-mediated cytotoxicity seen at 12 hr post-tuftsin-injection is primarily mediated by activated NK cells, while the augmentation observed at 24 and 264 hr is mediated by both activated NK cells and monocyte/macrophages.

Our *in vivo* results also showed a lack of dose dependency for the primary peak of augmentated activity although 50 μg animal tended to produce slightly greater stimulation for longer durations. Comparable levels of augmented murine natural cell-mediated cytotoxicity have been found following inoculation of a wide range of doses with various agents.[28-30] The results suggest that tuftsin, like other immunomodulators may effect the rapid *in vivo* boosting of natural cell-mediated cytotoxicity in an all-or-none fashion with complex regulating mechanisms. The secondary peak of augmented activity, however, was clearly dose dependent, and thus suggests a totally different activation pathway. We have previously shown that tuftsin has both mitogenic and colony-stimulating potential.[23] The secondary peak of tuftsin-augmented natural cytotoxicity at 11–12 days in spleen and bone marrow suspensions may thus represent the effecct of tuftsin on precursor populations that requires a maturation period to achieve cytotoxic capabilities. The activity of tuftsin to induce the maturation of noncytotoxic precursors into cytotoxic effectors is also reflected by the *in*

vivo effects of tuftsin on the bone marrow, which is believed to be the source of precursors for all naturally cytotoxic effector cells.

In summary, the results of this study have shown that synthetic tuftsin is a potent *in vitro* stimulatory agent for murine and human natural cell-mediated cytotoxicity. The tuftsin-induced enhancement of natural cell-mediated cytotoxicity appears to be manifested by the direct stimulation of monocytes, macrophages, granulocytes, and NK cells. We have also shown that tuftsin can function *in vivo* to substantially augment natural cell-mediated cytotoxicity. Since tuftsin is a physiological peptide and now has been shown to directly stimulate the naturally occurring tumoricidal capacities of monocytes, macrophages, granulocytes, and NK cells, its potential role as an immunotherapeutic agent against tumorigenesis indeed warrants extensive study.

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A Biochemical Study of the Phagocytic Activities of Tuftsin and Its Analogues

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Najjar *et al.*[1-3] showed that a cytophilic γ-globulin fraction, leukokinin, stimulated phagocytic activity of mammalian leukocytes, and the whole stimulatory effect of leukokinin could be ascribed to a single tetrapeptide fragment,[4,5] tuftsin, whose sequence was determined to be Thr-Lys-Pro-Arg.[6] Recently, many reports have appeared on the role of tuftsin and its analogues in phagocytosis of mammalian leukocytes and their antineoplastic effect. Tuftsin and its analogues were synthesized by a conventional liquid-phase method[7] and examined for their phagocytosis-stimulating activity. We have already reported that tuftsin and its analogues stimulate the phagocytosis of *Staphylococcus aureus* by guinea pig peritoneal exudate granulocytes.[8] The mechanism of action of tuftsin has not been explored in detail. Hydrogen peroxide is one of the bactericidal factors in leukocytes, and we can assess the bactericidal activity of leukocytes by the nitrobluetetrazolium (NBT) reduction assay instead of measuring hydrogen peroxide. Spirer *et al.*[9] found that tuftsin stimulates the NBT reduction of human polymorphonuclear leukocytes. Stabinsky *et al.*[10] reported that the mechanism of action of tuftsin is due to the increase in intracellular cGMP levels and the decrease in intracellular cAMP levels. 4[lys]-Tuftsin is one of our synthesized tuftsin analogues whose sequence is H-Thr-Lys-Pro-Lys-OH. It should be of interest to study the effect of 4[lys]-tuftsin on host immune responses to microbial infections. This paper deals with the effect of 4[lys]-tuftsin on *in vitro* and *in vivo* phagocytosis.

MATERIALS AND METHODS

In Vivo *Protective Effect of 4[lys]-Tuftsin against Microbial Infection in Immunosuppressed Mice*

Five-week-old male mice of the ICR strain that were injected intraperitoneally (i.p.) with 50 mg of cyclophosphamide per kg 4 days before infection were pretreated intravenously (i.v.) with 5–75 mg of 4[lys]-tuftsin per kg daily for 7 days. Twenty-four hours after the final pretreatment, the mice were challenged i.v. with 3×10^7 cells of *Candida albicans* and the mortality was recorded. On the other hand, ICR mice were orally or i.v. administered 1–250 mg of 4[lys]-tuftsin per kg daily for 7 consecutive

days. Twenty-four hours after the final administration, they were inoculated subcutaneously (s.c.) with sarcoma-180 cells (3×10^6/mouse) and challenged i.v. 24 hr later with 3×10^5 cells of *C. albicans*. The kidneys were removed on the 7th day after the infection and homogenized in 10 ml of phosphate-buffered saline (PBS). The homogenized suspension was diluted serially 10-fold with PBS and 0.1 ml of each dilution was spread on Sabouraud's dextrose agar. Three plates were prepared for each dilution of the individual specimens. Colonies were counted after incubation of the plates at 31°C for 20 hr. Each experiment was carried out three times.

Effect of Combination Therapy with 4[lys]-Tuftsin and Antibiotics

Five-week-old male mice of the ICR strain that were injected i.p. with 150 mg of cyclophosphamide per kg 4 days before infection were pretreated i.v. with 10 mg of 4[lys]-tuftsin per kg/day for 7 days. Twenty-four hours after the final pretreatment, the mice were challenged s.c. with 1×10^6 cells of *Pseudomonas aeruginosa* and then injected s.c. with the antibiotic 5 hr after infection. Deaths within 7 days after injection were recorded. The experiment was carried out three times.

Preparation of Normal Polymorphonuclear Leukocytes (PMNs)

Albino male rabbits were injected i.p. with 200 ml of 0.1% glycogen containing 10 units of heparin per ml, sacrificed 4 to 5 hr later, and the peritoneal cavity was washed with 200 ml of PBS containing 5 units of heparin per ml. The PMNs were collected by low-speed centrifugation at 800 rpm for 3 min. The PMNs were washed twice with PBS and suspended in Hanks solution to 10^7 cells/ml. All operations were done in an ice bath. The purity of the PMNs was more than 95%.

Preparation of Normal Macrophages (Mφs)

Albino male rabbits were injected i.p. with 80 ml of liquid paraffin and killed 3 to 4 days later, and their peritoneal exudate cells were harvested with 200 ml of PBS containing 5 units of heparin per ml. The liquid paraffin was removed with a siliconized separating funnel. After this procedure, the method was the same as that for the preparation of PMNs. The purity of macrophages was 70% to 95%, as determined by Giemsa staining.

In Vitro *Phagocytosis Experiment*

The PMNs or Mφs were incubated, at 37°C for 90 min with shaking, in Hanks solution containing 10% inactivated rabbit serum at a cell density of 1.0×10^7/ml, with or without 10–500 μg of tuftsin or 4[lys]-tuftsin per ml and 1×10^6 living cells of the Smith diffuse strain of *S. aureus* per ml were added. Immediately after addition of the bacteria, 2 ml of the cell suspension was centrifuged at 3500 rpm for 20 min. The sedimented cells were given a hypotonic treatment of 2 ml of water and allowed to stand for 15 min at room temperature. The survivors were counted by the plating method. The rest of the cell suspension with added bacteria was incubated at 37°C for 30 min with shaking and diluted with saline appropriately. The viable bacterial cells were counted by the plating method.

Assay of β-Glucuronidase[11,12]

After incubation with or without 4[lys]-tuftsin, a PMN suspension was incubated at 37°C for 30 min together with *S. aureus*, Smith diffuse strain, and then centrifuged at 3500 rpm for 20 min. The sedimented cells were treated hypotonically with water and the supernatant was obtained by centrifugation. To test tubes containing 100 μl of the supernatant, 100 μl of 0.2 *M* Na-acetate-buffered solution (pH 5.0) and 25 μl of 0.01 *M* Na-phenolphthalein β-glucuronide were added. The mixtures were incubated for 19 hr at 37°C. The reaction was stopped by addition of 1 ml of 0.5 *M* glycine-buffered solution (pH 10.5) and centrifuged at 3000 rpm for 5 min. Absorbance of the supernatant was measured at 550 nm in a Hitachi Spectrophotometer Model 200-20. Activity of β-glucuronidase was calculated from the amount of free phenolphthalein and the results were expressed as μg of phenolphthalein in 1×10^6 cells after 19 hr.

Assay of Superoxide Anion

This test was carried out accourding to the method of Richard *et al.*[13] Rabbit peritoneal Mφs were prepared as described above. 1×10^5 of the Mφs in 0.1 ml of Hanks solution were pipetted into test tubes containing 1.5 ml of a reaction mixture composed of 80 μ*M* ferricytochrome C (Sigma Chemical Co., St. Louis, MO, USA), 0.1 μg of phorbol myristate acetate (PMA: Sigma Chemical Co.,) per ml and 0.1-1.0 μg of 4[lys]-tuftsin in final concentrations. Control mixtures contained bovine erythrocyte superoxide dismutase (Sigma Chemical Co.) at a final concentration of 30 μg/ml in addition to one of the stimuli. After a 60-min incubation at 37°C, the reaction mixture was stopped by placing the tubes in ice for 5 min. The absorbance of the reaction mixtures was determined spectrophotometrically with mixtures from tubes with superoxide dismutase used as blanks, and the amount of cytochrome C reduced was determined by the equation $E_{550\,nm} = 2.1 \times 10^4\ M^{-1}\,cm^{-1}$.

Assay of [^{3}H]-Thymidine Uptake by Mouse Spleen Cells

Spleen cells were prepared from 5- to 6-week-old male BALB/c mice. One-ml quantities of mouse spleen cells (2×10^6 cells/ml) that were suspended in RPMI640 medium containing 10% fetal calf serum were pipetted into tubes and 1 μg of CoA and 10^{-3}–10 μg of 4[lys]-tuftsin per ml final concentration, were added. The mixtures were incubated at 37°C in 5% CO_2, 95% air for 48 hr and after addition of 0.5 μCi of [^{3}H]-thymidine, they were further incubated at 37°C for 24 hr and the spleen cells were harvested. [^{3}H]-thymidine uptake by the spleen cells was measured in a liquid scintillation counter.

RESULTS

Increased Resistance of 4[lys]-Tuftsin-treated Mice to Microbial Infections

The ability of 4[lys]-tuftsin to restore the defense mechanism against microbial infection in mice under treatment with the anticancer drug cyclophosphamide was studied. In the group not treated with cyclophosphamide, no mice died although, as

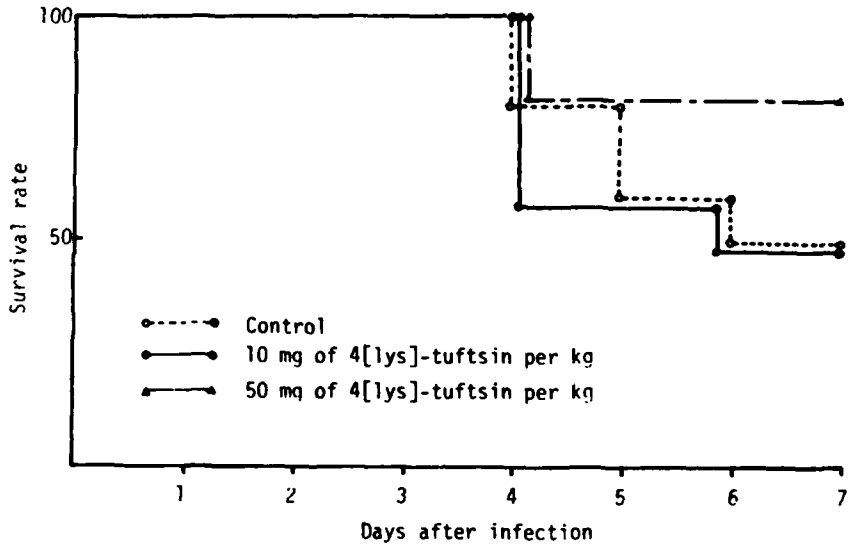

FIGURE 1. Effect of 4[lys]-tuftsin pretreatment (i.v.) on the lifespan of mice inoculated subcutaneously with cyclophosphamide and then challenged intravenously with *C. albicans*.

shown in FIGURE 1, in the treated control group, only 50% of the mice had survived 7 days after infection. However, the ratio of the surviving mice increased to 80% in the group pretreated with 50 mg of 4[lys]-tuftsin per kg. This intravenous pretreatment with 4[lys]-tuftsin was found to overcome the suppression of the protective mechanism induced by the cyclophosphamide.

Effect of Combination Therapy with 4[lys]-Tuftsin and an Antibiotic

Recently, chemotherapy against opportunistic infections in the immunocompromised host has become a clinical problem. Combination therapy with an immunosti-

TABLE 1. Effect of Combination Therapy with 4[lys]-Tuftsin and Antibiotics

Drug	Number of Survivors			
	1	3	5	7 days[a]
Control	40/40	20	18	18/40[b] (45.0%)
4[lys]-tuftsin 10 mg/kg	39/40	18	14	13/40 (32.5%)
CTX[c] 10 mg/kg	40/40	22	21	21/40 (52.5%)
CTX 10 mg/kg + 4[lys]-T 10 mg/kg	40/40	20	20	20/40 (50.0%)
CTX 40 mg/kg	40/40	22	22	21/40 (52.5%)
CTX 40 mg/kg + 4[lys]-T 10 mg/kg	40/40	27	24	24/40 (60.0%)
CB-PC[d] 5 mg/kg	39/40	19	18	18/40 (45.0%)
CB-PC 5 mg/kg + 4[lys]-T 10 mg/kg	40/40	24	23	22/40 (55.0%)
CB-PC 20 mg/kg	40/40	27	25	24/40 (60.0%)
CB-PC 20 mg/kg + 4[lys]-T 10 mg/kg	40/40	30	29	29/40 (72.5%)

[a]Days after infection.

[b]Survival ratio $\frac{\text{Number of surviving animals}}{\text{Number of animals tested}}$

[c]CTX, Cefotaxime.

[d]CB-PC, Carbenicilline.

mulator and an antibiotic was studied. As shown in TABLE 1, the effect of the combination of 4[lys]-tuftsin and an antibiotic was somewhat better than that of 4[lys]-tuftsin or antibiotic alone in immunosuppressed mice infected with *P. aeruginosa*.

Activation of Candidacidal Activity by Pretreatment with 4[lys]-Tuftsin

Mice were orally or i.v. administered 1–250 mg of 4[lys]-tuftsin per kg/day for 7 consecutive days. After s.c. inoculation of S-180 tumors, they were challenged i.v. with *C. albicans*. Their kidneys were removed 7 days after infection and viable fungal cells in the kidney were counted (TABLE 2). The number of fungal cells on the 7th day was $55.0 \pm 23.0 \times 10^2$/kidney and 12.6 ± 6.3–$22.0 \pm 9.2 \times 10^2$/kidney in the untreated mice and in those treated orally with 4[lys]-tuftsin, respectively. The number of fungal cells in 4[lys]–tuftsin-treated mice was reduced to 60–80% by oral treatment and

TABLE 2. Number of Fungal Cells in the Kidney 7 Days after Intravenous Infection with *C. albicans*

	Drug	Fungal Cells/Kidney
	Control	$55.0 \pm 23.0 \times 10^2$
	4[lys]-tuftsin	
p.o.[a]	5 mg/kg	20.0 ± 8.3[b]
	50	22.0 ± 9.2[b]
	250	12.6 ± 6.3[b]
	Control	$62.4 \pm 33.2 \times 10^2$
	4[lys]-tuftsin	
i.v.	1 mg/kg	80.0 ± 28.3
	10	18.4 ± 5.9[b]
	50	24.1 ± 13.3[b]

[a]Oral administration.
[b]Statistically significant, $p < 0.01$.

60–70% by i.v. treatment as compared with that in the controls. The difference is statistically significant.

Effect of 4[lys]-Tuftsin on in Vitro *Phagocytosis of PMNs and Mφs*

The stimulatory effect of 4[lys]-tuftsin on *in vitro* phagocytosis of PMNs and Mφs was studied. PMNs or Mφs obtained from albino rabbits were incubated at 37°C for 1.5 hr with shaking, either alone or mixed with 4[lys]-tuftsin. After incubation, living cells of *S. aureus*, Smith diffuse strain, were added to the mixture and incubated at 37°C for 30 min with shaking. The surviving bacterial cells were counted by plating. TABLE 3 shows the effect of tuftsin treatment on phagocytosis of the staphylococci. The phagocytic activity of Mφs was stimulated by incubation with 4[lys]-tuftsin. The increase in bactericidal activity caused by the addition of 10–500 μg of 4[lys]-tuftsin per ml was statistically significant. However, bactericidal activity of PMNs was not stimulated by incubation with tuftsin or 4[lys]-tuftsin, although the number of surviving bacteria was reduced after hypotonic treatment in every case. This suggests that a large amount of bactericidal substance was released from Mφs and PMNs and

TABLE 3. Number of Viable Staphylococcal Cells Incubated with Rabbit-derived Polymorphonuclear Leukocytes or Macrophages in Tuftsin- or 4[lys]-Tuftsin-treated Mice

	Drug	% Surviving Staphylococci[a] Before Hypotonic Treatment	% Surviving Staphylococci[a] After Hypotonic Treatment
PMN	Control	77.8 ± 11.3	1.50 ± 0.38
	4[lys]-tuftsin		
	10μ/ml	104.9 ± 15.8	1.15 ± 0.36
	100	100.8 ± 7.7	1.44 ± 0.49
	500	95.0 ± 10.6	4.45 ± 0.36
	Tuftsin		
	100 μg/ml	86.6 ± 15.0	1.05 ± 0.38
Mφ	Control	88.8 ± 6.7	38.6 ± 15.6
	4[lys]-tuftsin		
	10μg/ml	64.0 ± 16.0[b]	35.5 ± 25.9
	100	49.9 ± 6.4[b]	34.7 ± 12.2
	500	52.2 ± 1.9[b]	91.7 ± 27.5

[a] % surviving staphylococci = $\frac{\text{survivors of incubation with PMNs of M}\phi\text{s}}{\text{survivors of incubation without the cells}}$

[b] Statistically significant, $p < 0.01$.

caused cellular destruction. The release of the bactericidal substance was greater from PMNs than from Mφs and tuftsin was more effective than 4[lys]-tuftsin in releasing it from PMNs. In general, the antimicrobial activity of the PMNs or Mφs can be explained on the basis of two kinds of mechanisms.[14,21,22] One explanation is based on oxidative mechanisms and the other on nonoxidative mechanisms such as cationic protein and elastase. We studied the release of β-glucuronidase from PMNs incubated with 4[lys]-tuftsin to clarify the relationship between lysosomal enzyme and bactericidal activity. After preincubation with 4[lys]-tuftsin, rabbit peritoneal PMNs were incubated at 37°C for 30 min in the presence of *S. aureus*. After centrifugation, the sedimented cells were treated hypotonically with water. As shown in TABLE 4, the release of β-glucuronidase did not increase after hypotonic treatment in the presence of 4[lys]-tuftsin. Therefore it is suggested that there is no direct relationship between the bactericidal substance released from the cells after hypotonic treatment and lysosomal enzymes. The effect of 4[lys]-tuftsin on the release of superoxide anions (O_2^-) from

TABLE 4. The Relationship between Release of β-Glucuronidase by PMNs and Number of Surviving Staphylococci after Hypotonic Treatment of Polymorphonuclear Leukocytes after Incubation with 4[lys]-Tuftsin

Drug	Release of β-glucuronidase	% Surviving Staphylococci[a]
Control	6.8 ± 0.2 μg/ml[b]	1.29 ± 0.42
4[lys]-tuftsin		
10 μg/ml	6.1 ± 1.0	0.93 ± 0.36
100	6.7 ± 0.3	1.16 ± 0.50
500	7.4 ± 2.0	4.54 ± 0.36

[a] % surviving staphylococci = $\frac{\text{survivors of incubation with PMNs}}{\text{survivors of incubation without PMNs}}$

[b] μg of phenolphthalein/1 × 10^6 cells/19 hrs.

TABLE 5. Release of O_2^- by Rabbit Peritoneal Macrophages in the Presence or Absence of 4[lys]-Tuftsin[a]

Drug	Ferricytochrome C Reduction[b] $\times 10^{-6}$ *M*
Control	9.52
4[lys]-tuftsin	
0.1 μg/ml	11.90
0.5	11.67
1.0	10.47

[a]Number of cells: 1×10^5/ml.
[b]Reduction time: 60 min.

rabbit exudate cells was determined (TABLE 5). The release of O_2^- from Mφs was increased 10–30% by incubation with 0.1–10 μg of 4[lys]-tuftsin per ml but there was no effect of 4[lys]-tuftsin on the release of O_2^- from PMNs. Measurement of superoxide anion is one of the parameters used to determine the bactericidal effect of leukocytes, as well as NBT reduction. Therefore, it is suggested that 4[lys]-tuftsin stimulated the bactericidal activity of the Mφs.

Effect of 4[lys]-Tuftsin on [^{3}H]-Thymidine Uptake by Mouse Spleen Cells

[^{3}H]-thymidine uptake by mouse spleen cells stimulated with CoA in the presence or absence of 4[lys]-tuftsin was determined (FIG. 2). In the presence of 0.01–0.1 μg of 4[lys]-tuftsin per ml, [^{3}H]-thymidine uptake increased about 20%. From this result, 4[lys]-tuftsin appeared to increase cell-mediated immunity.

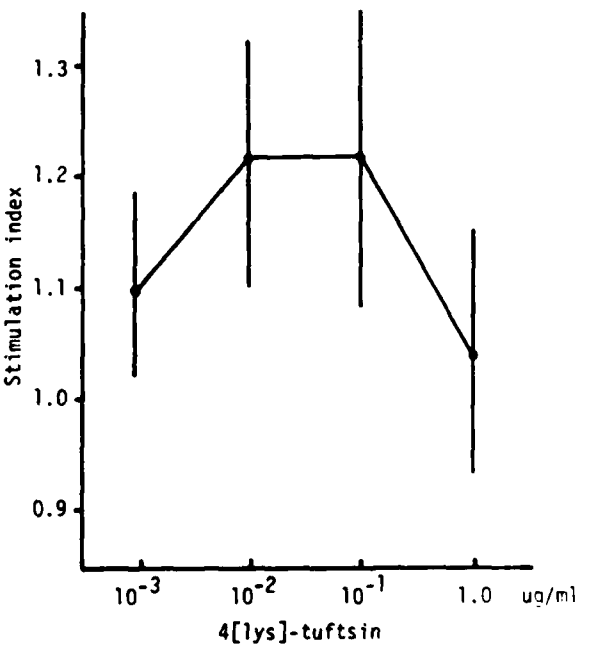

FIGURE 2. [^{3}H]-thymidine uptake by mouse spleen cells stimulated with CoA in the presence or absence of 4[lys]-tuftsin.

$$\text{Stimulation index} = \frac{\text{cpm: 4[lys]-tuftsin added}}{\text{cpm: not added}}$$

DISCUSSION

During the past decade, many reports[15-19] have appeared on the role of tuftsin and its analogues in phagocytosis of mammlian leukocytes and their antineoplastic effects. We synthesized tuftsin and its analogues by a conventional liquid-phase method and reported the *in vitro* phagocytosis-stimulating activity of the tuftsin analogues.[7,8] However, only a few articles have been published so far[18,20] dealing with the effect of tuftsin on the *in vivo* protective mechanism against pathogenic microbes. It is not clear whether *in vitro* experiments on phagocytosis reflect accurately the *in vivo* phenomena. To clarify this point, we studied the effect of 4[lys]-tuftsin pretreatment on microbial infection with *C. albicans* in mice. Intravenous treatment with 4[lys]-tuftsin overcame the suppression of protective mechanisms produced by cyclophosphamide (FIG. 1). Combination therapy with 4[lys]-tuftsin and an antibiotic was somewhat better than treatment with either one alone (TABLE 1). When 4[lys]-tuftsin was given before injection with *C. albicans*, marked suppression of bacterial growth in the kidneys of tumor-bearing mice (TABLE 2). The phagocytosis-stimulating activity of 4[lys]-tuftsin was confirmed by *in vitro* experiments. Phagocytic and bactericidal activity of rabbit peritoneal macrophages was increased by incubation with 4[lys]-tuftsin and the levels of bactericidal substance in PMN increased (TABLE 3). Biggar *et al.*[24] found that H_2O_2 had no bactericidal activity against *S. faecalis* in the vacuoles of rabbit lung macrophages and there was no increase in NBT reduction in the macrophages engulfing the bacteria. Superoxide anions in the vacuoles of macrophages did not show the bactericidal mechanisms of tuftsin and those authors found that incubation of human PMNs or mouse peritoneal Mϕs with tuftsin caused an increase in intracellular cGMP levels, accompanied by a decrease in intracellular cAMP. 4[lys]-tuftsin was also found to stimulate phagocytosis by leukocytes mildly. Further detailed investigation will be necessary to learn the mechanism of bactericidal activity of leukocytes incubated with 4[lys]-tuftsin. Najjar *et al.*[19] reported that tuftsin was capable of stimulating not only bactericidal activity but also tumoricidal activity. It has been found that the increase in antitumor activity caused by tuftsin is mainly due to stimulation of the activity of NK cells or macrophages (Phillips *et al.*,[20] Nishioka *et al.*[21]). It may be considered that 4[lys]-tuftsin stimulates antitumor activity as an immunomodulator, because incubation of mouse spleen cells with 4[lys]-tuftsin caused a slight increase in [^{3}H]-thymidine uptake. From these results, 4[lys]-tuftsin is expected to be useful for treatment of patients with malignant tumors by using it together with an antibiotic because it induces regression of tumor cells and prevents terminal infections.

SUMMARY

The effects of tuftsin and one of its analogues (4[lys]-tuftsin) on phagocytosis of *Staphylococcus aureus* and *Candida albicans* were investigated in mice and rabbits. Mice were intravenously or orally administered 1–25 mg of 4[lys]-tuftsin per kg daily for 4 to 7 days. After the mice were further treated with cyclophosphamide, they were intravenously challenged with *C. albicans*. More than 50% of the mice infected with *C. albicans* were killed within 7 days, although only 20% to 40% of those infected with the same microbes after 4[lys]-tuftsin treatment died. A combination of 4[lys]-tuftsin and an antibiotic was found to be somewhat more effective than either one alone. Further, pretreatment with 4[lys]-tuftsin depressed microbial growth in the kidneys of mice bearing S-180 tumors. Rabbit peritoneal macrophages and polymorphonuclear leuko-

cytes were harvested by intraperitoneal injection of liquid paraffin and of glycogen solution, respectively. For *in vitro* study of phagocytosis, rabbit macrophages of polymorphonuclear leukocytes were incubated in Hanks solution together with *S. aureus* and the number of survivors was determined. Enhanced engulfing activity of macrophages and increased bactericidal activity of polymorphonuclear leukocytes were shown by the *in vitro* phagocytosis experiments. It is expected that 4[lys]-tuftsin will be effective against infectious disease, especially in immunocompromised hosts such as patients with malignant tumors.

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Congenital Tuftsin Deficiency

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Resistance to bacterial infection is a complex phenomenon involving interactions between a number of cellular and humoral factors. Phagocytosis and intracellular killing of bacteria are important functions of polymorphonuclear leukocytes (PMN).[1] The normal pathways of phagocytosis are: (a) opsonization of bacteria by IgG and complement components; (b) engulfment of opsonized bacteria by neutrophils in the presence of the tetrapeptide tuftsin; and (c) formation of the phagocytic vacuole and degranulation in which granules pour their contents into vacuoles and digest bacteria.[1,2]

During the past few years, several reports have appeared describing patients exhibiting deficiency in the phagocytic activity of the blood neutrophilic leukocytes. These abnormalities can be separated into three general types of functional deficiencies.[1,2] The first is a deficiency of opsonizing serum factors.[3,4] The clearest examples of these are deficiencies of serum complement factors such as C_3 and C_5. All these factors involve the opsonization of the target particles. The second is a deficiency of the tetrapeptide tuftsin,[2,5,6] which represents an impairment of the engulfing activity of the blood neutrophil. Tuftsin acts directly on the phagocyte and not on the target particle. The third is a deficiency in bactericidal activity, which is expressed in the inability of the phagocyte to destroy the ingested organism, such as in cases of chronic granulomatous disease.[7]

This paper will present five patients with congenital tuftsin deficiency, and tuftsin activity in the serum of full-term and premature newborns. Newborns were selected for this study because it is well known, although poorly understood, that newborn infants have high susceptibility to bacterial diseases.[8]

MATERIALS AND METHODS

We have studied tuftsin activity in four different pediatric groups. In the first group, 20 premature infants (11 males, 9 females) at 30–33 weeks of gestation were studied. The birth weights ranged from 1,300 g to 2,250 g. The second group consisted of 20 full-term infants (13 males, 7 females) at 36–42 weeks of gestation. The birth weights ranged from 2,350 g to 3,750 g. The third group comprised 20 healthy children (10 males, 10 females) 7 to 15 years old and served as a control (TABLE 1). In the fourth group, we present all patients who have been described in the international literature as having congenital tuftsin deficiency.

Blood samples were drawn from babies born at Alexandra Maternity Hospital within the first day of life. The controls were seen as outpatients at Aghia Sophia Children's Hospital. The serum from each individual was stored at −40°C until assayed.

The tuftsin level of serum activity was assayed briefly as follows:[9-11] γ-globulin was prepared from inactivated and deopsonized serum by precipitation with saturated ammonium sulfate and dialyzed against 0.1 *M* phosphate buffer, pH 8.1. Ten mg of

TABLE 1. Clinical Description of Subjects Used in Phagocytosis Studies

Subjects	Number	Age	Sex F	Sex M	Gestational Age (Wk)	Birth Weight (g)
Premature infants	20	1 day	9	11	30–33	1,300–2,250
Full-term newborns	20	1 day	7	13	36–42	2,350–3,750
Normal children	20	7–15 yr	10	10	—	
Patients with congenital tuftsin deficiency	5	3/12–15 yr	3	2	—	

γ-globulin were then digested with 0.5 mg of trypsin. The released tuftsin, if any, was extracted in ethanol. The ethanol extract was evaporated to dryness and taken up in 0.5 ml of Krebs-Ringer phagocytosis medium and tested for its ability to stimulate phagocytosis of *Staphylococcus aureus*. The bacteria were thoroughly opsonized with normal inactivated serum. An aliquot of 0.1 ml, representing 2 mg of the original γ-globulin, was used as the source of tuftsin. Throughout this study, phagocytic cells were prepared from the buffy coat of freshly drawn heparin-treated human blood. All assays were run in duplicate.

The extent of phagocytosis is recorded as the phagocytic index. This represents the number of cells containing one or more bacteria per 100 PMN neutrophils counted. Phagocytic stimulation is the phagocytic index obtained in the presence of tuftsin minus the reagent control. No less than 400 PMN cells were counted by two different observers.

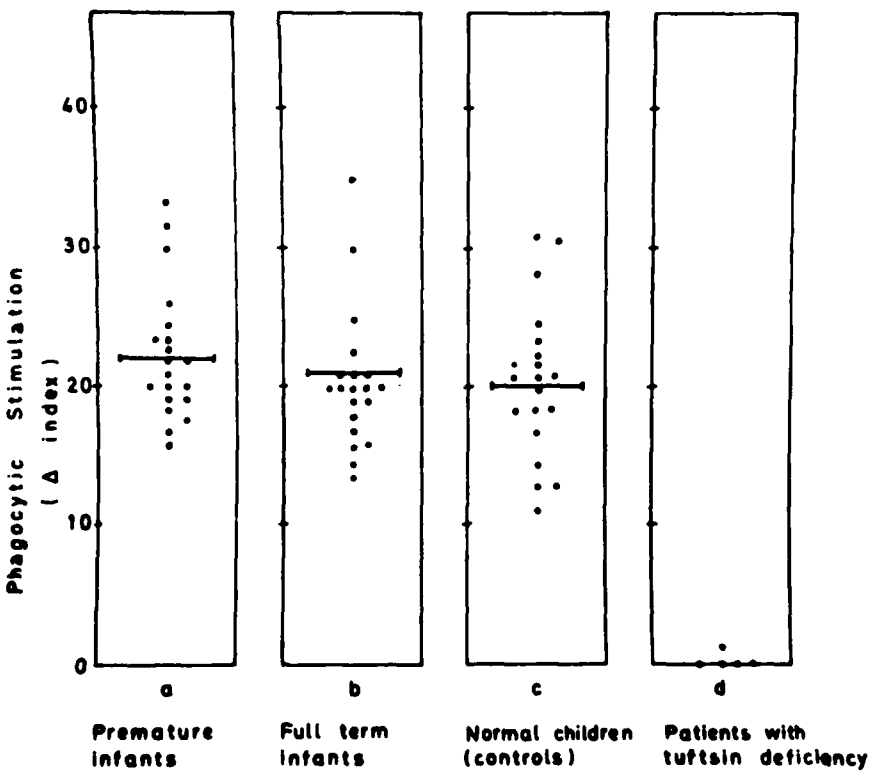

FIGURE 1. Serum tuftsin activity in four different pediatric groups: (a) premature infants, (b) full-term infants, (c) normal children (controls), and (d) patients with tuftsin deficiency.

RESULTS

FIGURE 1 shows the results obtained in premature and full-term newborns, normal children, and patients with congenital tuftsin deficiency. The variation in the values obtained does not relate to the age or sex or birth weight of the individual. The last group showed complete absence of tuftsin activity. Five families with familial tuftsin deficiency have been reported in the United States and Japan. In each case, there has been a history of recurrent infection, very severe in the newborn period and early childhood but becoming milder as the patient grows up. The major symptomology is primarily the result of widespread infections. These involve the low respiratory system (bronchitis, bronchiolitis, and pneumonia), the skin (seborrheic dermatitis and/or pyodermia), and lymph nodes (usually draining lymphadenitis) (TABLE 2). The most frequent infecting organisms were *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Candida albicans*.

It was of interest to study possible deficiencies of other members in the families of patients with congenital tuftsin deficiency. Indeed, one parent and a sibling of each family, had symptomless tuftsin deficiency (TABLE 3).

The laboratory finding are shown in TABLE 4. The negative findings are no less important than the positive ones. The cellular and humoral immunity was normal. Serum proteins and immunoglobulins (γG, γM, γA) are within normal values. Fractionation of the patients γ-globulin on phosphocellulose columns gave a normal

TABLE 2. Symptomology of Patients with Congenital Tuftsin Deficiency

1. Respiratory system	Bronchitis, bronchiolitis, pneumonia
2. Throat—Ears	Tonsilitis, otitis media
3. Skin	Seborrheic dermatitis, pyodermia
4. Lymph nodes	Lymphadenitis, often purulent
5. Blood	Septicemia

pattern in each of the four peaks. Tuftsin activity as measured by digestion with both PMN membrane enzyme leukokinase or trypsin proved to be absent in the patients' serum. C_3 and C_5 also gave normal values. The blood PMN leukocyte count is elevated, but the cells are capable of reducing nitro-blue tetrazolium to a normal extent.

In all cases that have been studied so far, including those reported in Japan, γ-globulin studies show that the patient's tuftsin is replaced by a mutant peptide that proved strongly inhibitory to the stimulatory activity of the normal tetrapeptide tuftsin, Thr-Lys-Pro-Arg. In one such patient who had been studied extensively, the isolated tetrapeptide appears to represent a mutation where the lysine residue has been replaced by a glutamic acid residue with the tentative structure Thr-Glu-Pro-Arg. The inhibition is overcome by an excess of normal tuftsin.[12]

The administration of γ-globulin intramuscularly 1–2 g is followed quickly by dramatic improvement lasting a few weeks. A second injection would alleviate the disease for another period of several weeks. The response to γ-globulin sets this syndrome quite apart from other deficiencies that affect the phagocytic activity of the PMN.

DISCUSSION

Several syndromes have recently been described relating to impaired phagocytosis.[13] Our results show that premature and full-term newborns have normal tuftsin

TABLE 3. Tuftsin Activity in the Serum of Five Families Having Patients with Tuftsin Deficiency

	1. American			2. American			3. American			4. American			5. Japanese		
Subject	Sex	Age (yr)	Phagocytic Stimulation (Δ index)	Sex	Age (yr)	Phagocytic Stimulation (Δ index)	Sex	Age (yr)	Phagocytic Stimulation (Δ index)	Sex	Age (yr)	Phagocytic Stimulation (Δ index)	Sex	Age (yr)	Phagocytic Stimulation (Δ index)
Patient	M	10/12	0	F	15	0	F	3	0	M	6	1	F	3/12	0
Father	M	30	0	M	52	20	M	47	23	M	48	0	M	--	0
Mother	F	20	21	F	50	0	F	42	0	F	40	19	F	—	20
Sibling 1	M	50	20	F	7	23	M	18	2						
Sibling 2				F	15	17	M	4	17						
Sibling 3				F	20	19	M	8	19						
Sibling 4				M	18	20	M	12	18						
Sibling 5							M	16	24						
Sibling 6							M	21	21						
Sibling 7							F	2	19						
Sibling 8							F	6	18						
Sibling 9							F	10	20						
Sibling 10							F	11	25						

activity, although the splenic function of the babies is diminished and produces γ-globulin in minute amounts.[13] However, levels of γ-globulin at birth approach levels found in normal adults as a result of the passage of maternal γ-globulins across the placenta during the second half of pregnancy. The babies fully synthesize γ-globulins after the third to fourth week of life. These findings are an indication that the leukophilic γ-globulin of the babies is maternal in origin.

During the past few years, several reports from Najjar's laboratory have shown that there are highly specific leukophilic γ-globulins that bind only to blood phagocytes,[10,14] polymorphonuclear cells, and monocytes. This leukophilic γ-globulin has been termed leukokinin because it stimulates the phagocyte to increase its motility as well as its phagocytic activity. The leukokinin molecule, which is activated by the spleen, binds on the outer surface of the PMN cell membrane. The enzyme leukokinase, on the outer surface of the cell membrane, cleaves off the tetrapeptide to make it available to the cell.[2,10]

Two types of tuftsin deficiency have been found: acquired due to restriction of splenic function[9,15] and congenital due to various anomalies. These anomalies fall into

TABLE 4. Laboratory Findings of Patients with Congenital Tuftsin Deficiency

Test Taken	Remarks
Serum tuftsin activity	Absent or reduced
Peptide extracts	Inhibitory to normal tuftsin
Absolute number of PMN	Increased or normal
Immunoglobulins (γG,γM,γA)	Normal
Quantitative values of C_3 and C_5	Normal
Fuctional activity of C_3	Normal or reduced
Opsonins	Normal
NBT test	Normal
Isolated organisms	*S. aureus, Streptococcus pneumoniae, Candida albicans*
Administration of γ-globulin	Infection alleviated

four possible types:

Type I: Alteration of tuftsin structure or its deletion.
Type II: Inactivation or absence of the leukokinase enzyme of the phagocyte membrane.
Type III: Inactivation of the splenic enzyme or asplenia.
Type IV: Absence of sialic acid on the phagocyte membrane.

Up to the present we have identified only Type I of the four types of congenital tuftsin deficiency.[2,5,6] This type is the easiest to identify. A high level of suspicion would be aroused if clinical manifestations suggestive of the deficiency are present that respond favorably to intramuscular injections of γ-globulin. The injected γ-globulin merely represents a supply of normal and functional leukokinin, the parent carrier molecule of active free tuftsin.

It is clear from the preceding that *in vivo* tuftsin performs a unique function in combating infection. The clinical picture of congenital tuftsin deficiency is repeated and often severe infections. However, the severity of infection varies considerably. It was of interest to find that in every case of congenital tuftsin deficiency, one of the parents exhibited the defect as well as one or more children in the same family. Other than the fact that this syndrome is familial, it is difficult to evaluate its genetic characteristics. It is also clear that the symptomology is quite variable. Several subjects

who clearly had laboratory evidence of tuftsin deficiency did not show any symptoms. Other host factors must therefore, play a role.

The clinical picture, the negative routine laboratory findings, and the response to γ-globulin are the main characteristics of patients with tuftsin deficiency. The diagnosis can be confirmed by measuring tuftsin activity. The assay of tuftsin can be performed either by radioimmunoassay[16] or by biological activity of serum.[2] The observations that recurrent infections can best be alleviated through the administration of γ-globulin prompts the recommendation of periodic γ-globulin administration as a preventive measure for these patients. When tuftsin becomes available to the medical profession, its administration may prove essential as replacement and preventive therapy in cases of tuftsin deficiency.

SUMMARY

The serum tuftsin activity in four different pediatric groups has been studied: (a) 20 premature infants, (b) 20 full-term infants, (c) 20 normal children (controls), and (d) 5 patients with recurrent and severe infections of the respiratory system, skin, and lymph nodes. The normal children as well as the premature and full-term infants had normal tuftsin activity. Tuftsin activity in the serum of the 5 patients in group (d) was absent. Cellular and humoral immunity in these patients was normal. All patients have been shown to have a mutant peptide that is strongly inhibitory to the normal tetrapeptide tuftsin. The clinical response of these patients to γ-globulin was excellent.

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Acquired Tuftsin Deficiency

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Galen's declaration that the spleen is an organ full of mystery continues to be appropriate despite the passage of time and advances in medicine. For centuries it was believed that the spleen is not physiologically essential and that removal of the spleen does not lead to any harmful consequences. During the last decades, splenectomy has been widely used as the treatment of choice for both trauma and various hematological disorders.

This approach, termed by Morgenstern[1] "the surgical inviolability of the spleen," was challenged by King and Schumacher in 1952.[2] They published their observation that splenectomy performed in infants exposes them to serious bacterial infections. This observation focused interest on the role of the spleen in immunity and infection. Overwhelming sepsis later has also been reported in adults even many years after splenectomy.[3] The incidence of sepsis after removal of the spleen for trauma (0.5–1%) is considerably lower than that following elective splenectomy. It is today widely accepted that the increased susceptibility to fulminant infections of splenectomized patients is a result of detrimental consequences of removal of an organ of major importance in maintaining defence mechanisms against bacteria. Not all immune functions were sufficiently clarified, but it was agreed that the spleen plays a particularly important part in controlling the phagocytic capacity of polymorphonuclear leukocytes.

This was first suggested in an elegant and precise work documented by Najjar and his group from Tufts University.[3]

They have found defective phagocytosis in the leukocytes of humans and animals who have undergone splenectomy and attributed this defect to the absence of tuftsin, a basic tetrapeptide (L-Thr-L-Lys-L-Pro-L-Arg) produced or released in the spleen, that acts as a phagocytosis-stimulating agent of both polymorphonuclear leukocytes and macrophages.[4]

Tuftsin has been synthesized and the biological activity of the synthetic substance and its equivalence to the natural peptide have been shown *in vitro* and *in vivo* by various assays[5,6] and in various systems.[7]

We have developed a radioimmunoassay for measuring serum tuftsin concentra-

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tions,[8] and we report here the results of our evaluation of tuftsin concentrations in a large group of patients who had undergone splenectomy for various reasons and in a smaller group of patients with clinical situations in which the spleen functions below the normal level for its size, even when it is enlarged.

PATIENTS AND METHODS

We studied:

1. 35 healthy medical and nursing students (21 men and 14 women) aged 19–35 years, and 21 healthy children aged 18 months–10 years (11 boys and 10 girls).
2. 128 patients from the Municipal Governmental Medical Centre, Tel Aviv University, and the Zentrum der Innern Medizin, Goethe Universität Klinikum, Frankfurt, who had undergone splenectomy one to 28 years earlier. This group of patients included 50 who had had traumatic splenectomy, 20 who had had elective splenectomy, 38 with Hodgkin's disease who had had splenectomy, and 20 with other malignant disorders, who had had splenectomy. A further 14 patients with Hodgkin's disease were also studied before they underwent splenectomy and four with miscellaneous conditions had their tuftsin concentrations measured before and after splenectomy.
3. 21 patients (14 with homozygous SS disease, 5 with Hb SC disease and 2 with CC disease). The patients were betweeen 3 and 21 years, neither infected nor in crisis at the time or during the 6-month period preceding blood collection. The sera were collected in St. Petersburg, Florida, stored at −20°C and shipped frozen to our laboratory.
4. 6 children aged 3–10 years with a previous history of severe protein-calorie malnutrition in their early infancy (2–5 mo) due to intractable diarrhea without history of recurrent infections, and in good health at the time of examination or during the last months preceding the blood collection.
5. 3 infants diagnosed as suffering from gluten-induced enteropathy on clinical basis only (without biopsy) and 4 adolescents with proven ulcerative colitis – both groups during the acute stage of the disease only.
6. 8 cord sera from uncomplicated pregnancy and delivery terminated by full-term healthy infants. Serum tuftsin was determined by radioimmunoassay as previously described.[8] Splenic scans were performed with *methyl*-^{99m}Tc sulfur colloid.

RESULTS

The amounts of material immunochemically related to tuftsin found in the various sera are shown in FIGURES 1, 2, and 3.

Statistical examination (Student's t-test) showed a significant difference ($p < 0.001$) between concentrations in the normal subjects and those in the patients who had undergone splenectomy electively both for various hematological disorders or because of Hodgkin's disease or other malignancies. There was no significant difference in concentration between the normal subjects and those who had had a traumatic splenectomy. Similarly, patients who had had an elective splenectomy for Hodgkin's disease and those who had had one for other causes did not differ significantly in their tuftsin concentrations.

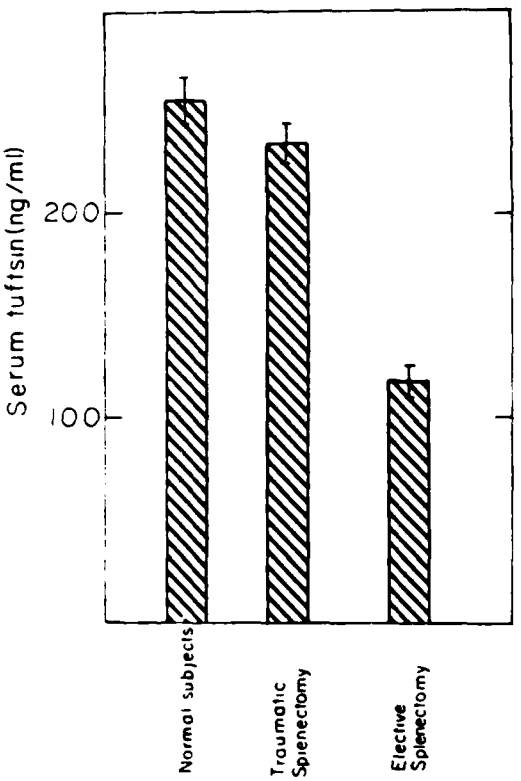

FIGURE 1. Serum tuftsin concentration in normal subjects, traumatic splenectomy, and elective splenectomy.

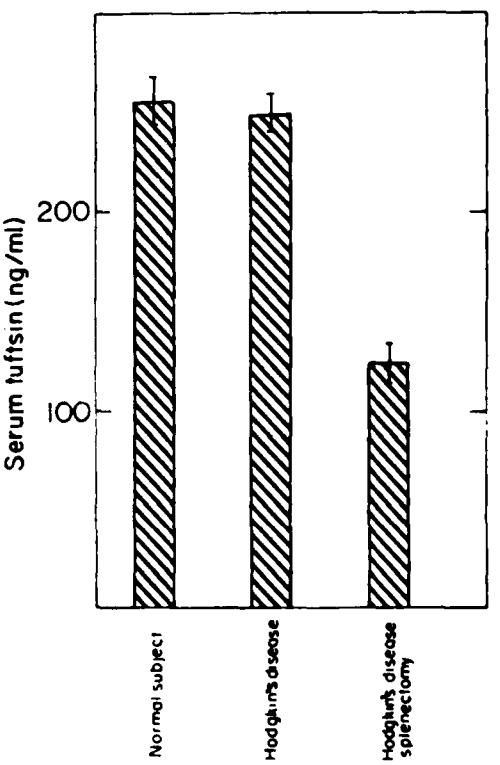

FIGURE 2. Serum tuftsin in Hodgkin's patients without or before splenectomy and after splenectomy.

In patients who were studied immediately after Hodgkin's disease had been diagnosed and before splenectomy was performed, the mean tuftsin concentration was not significantly different from normal. There were no differences in tuftsin concentration between adults and children. In the 26 patients studied for presence or absence of splenic tissue by *methyl*-^{99}Tc scan (FIGURE 4) the group I (14 patients: 8 traumatic, 5 incidental, 1 elective) tuftsin level concentration was significantly higher (and near the normal mean) than in group II with negative scan. The difference between the two groups was statistically significant ($p < 0.0001$).

In the patients with sickle cell disease, the mean (±SD) serum tuftsin concentration was significantly lower in patients with hemoglobin SS disease (154.3 ± 35.1 ng/ml, $p < 0.01$) and in patients with hemoglobin SC and hemoglobin CC disease (180.9 ± 42.7 ng/ml, $p < 0.05$) than in healthy controls (228.7 ± 46.7 ng/ml).

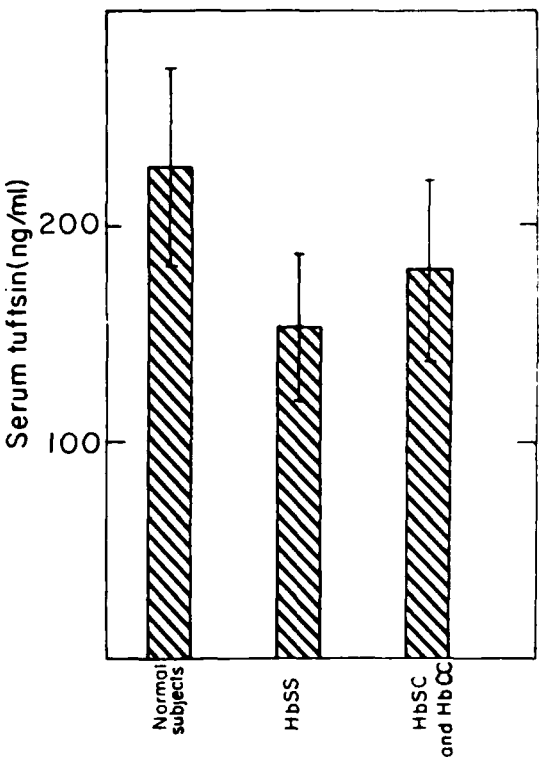

FIGURE 3. Serum tuftsin in patients with sickle cell disease (SCD).

The mean (±SD) serum tuftsin concentration in hemoglobin SS disease patients with Howell-Jolly bodies in their peripheral blood smears was significantly lower than in those without Howell-Jolly bodies but not related to the degree of splenomegaly.

In the children after protein-calorie malnutrition, the mean ±SD tuftsin concentration was 152 ± 27.3 ng/ml, significantly lower than in control children. The same was true for the infants with celiac disease and ulcerative colitis ($p < 0.001$).

All four patients who were studied before and after splenectomy showed a considerable fall in tuftsin concentrations after the operation. Two patients with spherocytosis had initial concentrations of 261 and 235 ng/ml that fell to 112 ng/ml and 87 ng/ml, respectively, after splenectomy. A patient suffering from an epidermoid splenic cyst had a normal value of 252 ng/ml before the operation and a value of 45 ng/ml two weeks after splenectomy.

A 7-month-old infant in whom a spleen torsion was diagnosed had a tuftsin

concentration of 98 ng/ml three days after the incident (and before splenectomy) and two weeks and two months after splenectomy showed values of 50 ng/ml and 40 ng/ml, respectively.

The amounts of material immunochemically related to tuftsin in cord blood samples was significantly higher than in the mother's serum or normal controls ($p < 0.001$).

DISCUSSION

Since Najjar's discovery of tuftsin,[4] it has been well documented that except for the rare syndromes of congenital tuftsin deficiency, the main problem is acquired tuftsin deficiency in splenectomized patients or in functional hyposplenic states.

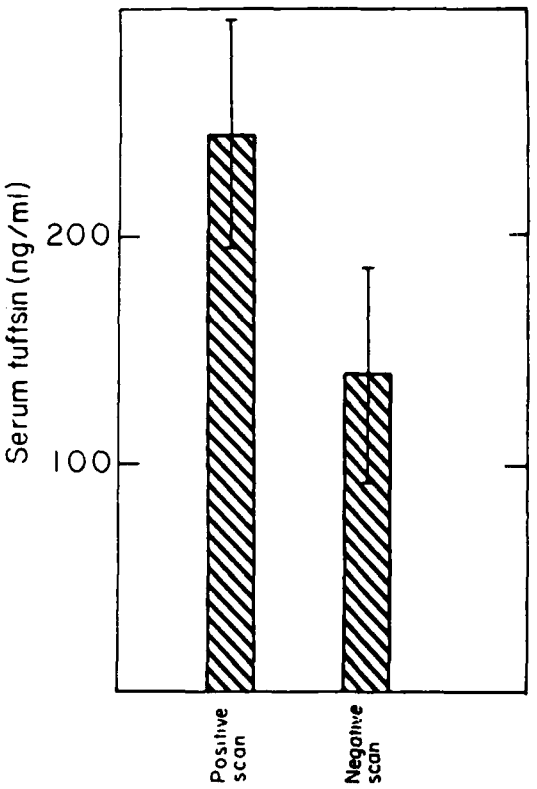

FIGURE 4. Correlation between serum tuftsin levels and *methyl*-^{99}Tc scan findings.

The fact that tuftsin concentrations are lower and the risk of fulminant infections is higher after elective splenectomy than after traumatic splenectomy may suggest that tuftsin plays an important role in the defense against bacterial infections. The relatively normal tuftsin concentrations found in our traumatic group may have been accounted for by Najjar's suggestion[5] that splenic tissue is implanted in the abdomen during the spleen rupture. In our study, normal tuftsin levels characterized almost all patients in whom scans showed splenic tissue activity; trauma was the most common cause of splenectomy in this group. On the other hand, low levels of tuftsin correlate well with negative scans.

Pearson,[9] using a *methyl*-^{99}Tc sulfur colloid scan, showed splenic tissue activity in a

group of children previously splenectomized because of injury and introduced the term "the born-again spleen."

The recurring splenic tissue activity in the patients who underwent splenectomies may be due to splenosis and/or accessory spleens. Splenosis is the term introduced in 1939 to describe the condition of multiple peritoneal implants of splenic tissue after severe trauma to the spleen. Disruption of the splenic capsula causes fragments of splenic pulp to the seeded throughout the peritoneal cavity, their number varying from several to hundreds. The auto-transplanted nodules provide their blood supply by small new arteries penetrating the capsula, which is derived from surrounding reactive granulation and fibrous tissue.

Accessory spleens are usually few in number and they differ from splenosis in that they are like miniatures of the main spleen and receive their blood supply through a small branch of the splenic artery. They have a real capsule containing smooth muscle and elastic tissue components, all of which are absent in splenosis.

Previous reports have attributed positive splenic scans exclusively to splenosis. Although it cannot be excluded without a laparotomy that accessory spleens may account for splenic function in some patients, our contribution supports the view that splenosis is the important element.

Is the evidence of returning splenic function an expression of required resistance to infection? Overwhelming sepsis has been reported in patients who underwent splenectomies with splenosis or remaining accessory spleens although with low incidence and in all these cases, a very small amount of splenic tissue was found. It is proposed that at least two factors are important to protect against infection—the volume of the splenic tissue and the presence of normal blood supply. The minimal critical mass of splenic tissue that is necessary for this purpose is still unknown.

The serum tuftsin concentrations in our patients with Hodgkin's disease is responsible for severe fulminant bacterial infections—infections that are infrequent in nonsplenectomized Hodgkin's patients. Our results suggest that the practice of performing staging laparotomy and splenectomy should be reconsidered.

A number of states with functional hypersplenism were described including sickle cell disease (SCD), congenital cyanotic heart disease, celiac disease, ulcerative colitis, immune complex disease, the newborn and preterm infant.[10] We have had the opportunity to quantitate tuftsin levels in some of these states.

Overwhelming infections contribute significantly to morbidity and mortality in patients with SCD. Although several abnormalities of host defense have been shown in SCD patients,[11-13] functional and eventually anatomic asplenia is likely to be an important factor in the pathogenesis of the increased susceptibility of these patients to systemic infection. The present data indicate that measurement of serum tuftsin levels may well be of value as an indicator of the presence of a defective splenic function in SCD patients. Indeed the lowest mean serum tuftsin concentration was recorded in patients with hemoglobin SS disease. In half of these patients, Howell-Jolly bodies, a reliable indicator of the presence of splenic hypofunction, were observed on peripheral blood smears. Our findings are in good agreement with those of Najjar,[14] who measured serum tuftsin activity in SCD patients using a different method.

In children after protein/calorie malnutrition, tuftsin levels were determined within the frame of a study on immunological functions in children years after recovery from protein/calorie malnutrition in their early infancy.[15] In the light of decreased levels of IgM, as in the postsplenectomy state, and the reduced concentration of serum tuftsin, our findings may indicate a late effect of splenic atrophy during PCM.

There is indirect evidence to support the idea of transient functional asplenia in the newborn period.[10] This was the reason for the tuftsin determination in the cord blood. Unfortunately, the group is too small to give rise to conclusions. The high level may be

a result of cross reactivity of other substances in the cord blood that react immunochemically like tuftsin.

Improvements in the technique of measuring splenic activity in this age group are required to provide additional information.

Recognition of the high risk of infection in asplenic patients and immediate diagnosis and treatment can be life saving.

There are promising approaches to preventing the risk of severe infections in asplenic patients. Immunization with pneumococcal polysacharide vaccines are generally indicated and effective, but may not be effective in children under 2 years of age. Prevention of infection by synthetic tuftsin administration holds promise for the future in both congenital and acquired tuftsin deficiency. But unlike congenital, acquired tuftsin deficiency is a preventable disorder. Whenever possible, alternatives to splenectomy should be considered. These alternatives include: nonoperative management of splenic injury, suture of ruptured spleen, prevention of splenic tissue *in situ* (like accessory spleens), hemisplenectomy, partial splenectomy, reconsideration of the need of staging laparotomy and splenectomy in lymphatic malignancies and postponement of splenectomy as long as possible in hematological disorders.

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Tuftsin Stimulation of Phagocytosis by the Retinal Pigment Epithelium

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The retinal pigment epithelium (RPE) is a phagocytic tissue. It is closely associated with the retina and is derived from the embryonic neuroectoderm. The RPE phagocytoses an estimated 10% by volume of the outer segments of the photoreceptors daily.[1] This clearly makes the RPE one of the more active of phagocytic tissues.

Anatomically the RPE is juxtaposed to the photoreceptors of the neural retina. It is a cuboidal to low columnar epithelium that has apical processes that interdigitate with and invest the outer segments of the photoreceptors. Phagocytosis occurs as the tips of the outer segments of the rod photoreceptors (ROS) are shed into the intercellular space.[1-3] The RPE recognizes, attaches to, surrounds, and ingests the effete packets of outer segment discs and degrades the resultant ingested rod outer segments (phagosomes) over several hours.[1,4] This is a circadian activity, and is triggered by the onset of light.[2] This extremely active function of the RPE was first described by Young in 1967.[3]

Macrophages are derived not from the neuroectoderm but from the embryonic mesoderm. That tuftsin stimulates phagocytosis by macrophages and leukocytes is clear.[5-7] Although several inhibitors of phagocytosis by the RPE have been reported,[8,9] no pharmacological stimulator of phagocytosis by the RPE has yet been described. The object of the research reported in this paper is to ascertain whether tuftsin can stimulate a tissue that is known to be phagocytic, but not derived from the embryonic mesoderm. We conclude from our observations that tuftsin is able to stimulate phagocytosis by the RPE.

MATERIALS AND METHODS

In Vivo

Albino rats of the Sprague-Dawley strain were obtained from Charles River laboratories and entrained to a 12:12, light:dark cycle for 20 days. To prevent retinal damage, the intensity of light in the rats' cages did not exceed 2 foot-candles.

One hour before the normal onset of light on the day of the experiment, the 40-day-old rats were given an intraocular injection of 2 microliters of a balanced salt solution (BSS)[a] or of BSS containing tuftsin (100 μg/ml). The vitreous volume is estimated to be 35 μl.[10] This results in an approximate intraocular concentration of 6 μg/ml.

The eyes were injected without exposing the animals to light by using an infrared viewer. Accurate placement of the needle was assured by direct visualization of the

[a]Balanced Salt Solution, ALCON.

needle in the eye. A 30-gauge needle was used with a polyethylene protector that allowed only 1 mm of needle to enter the eye. The site of injection was at the dorsal equator of the globe.

Lights were turned on at the usual time and one hour later, at the normal peak of phagocytosis, the rats were killed with an overdose of sodium pentobarbital. The eyes were enucleated and prepared for histology. The anterior structures of the eyes were removed by hemisection, and the remaining posterior portion of the eye was fixed in trialdehyde. Four hours later, the eyes were partially dehydrated using alcohols and immersed overnight in 2-hydroxypropyl methacrylate. The following day, the eyes were oriented and embedded in a fresh change of methacrylate, then polymerized with ultraviolet light. Sections two microns thick were taken of the central portion of the retina and RPE and stained with Lee's stain (FIGURE 1). The slides were examined using phase microscopy and the number of phagosomes larger than one micron were counted. Phagosomes were counted in terms of the number of phagosomes per microscopic field. Subsequently the number of phagosomes was calculated per 100 μm of RPE length.

In Vitro

Rats used for the *in vitro* experiments were of the Royal College of Surgeons strain without retinal degeneration.[11] These animals have normal retinas, but are congeneic with a strain of rats that have a congenital anomaly of retinal degeneration.[12-15]

Rats, 30-40 days old, were kept in a 12:12 light cycle until the day before the experiment. The evening before the experiment, the rats were put into constant light to block shedding of the outer segments.[16] The following morning, the rats were killed and the eyes enucleated. The eyes were hemisected and the posterior segment flattened. Each preparation was trimmed using a 4-mm trephine and the neural retina was peeled from the pigment epithelium using a millipore filter. The resulting disc was then quartered and each quarter placed with the RPE side uppermost in the well of a culture plate. Specimens were incubated in culture media of MEM with Earle's balanced salt solution with 2 m*M* l-glutamine, 20% fetal calf serum (FCS), and antibiotics for one hour. At the end of one hour, the medium was replaced with an identical medium containing fluorescent latex microspheres[b] 1.2 μm in diameter (10^7/ml). One half of the cultures also received tuftsin to obtain a concentration of 25 μg/ml. Six hours after adding the microspheres and tuftsin, the cultures were washed and prepared for histology using the methacrylate embedding techniques described above. Methacrylate, a water-soluble embedding medium, was used because microspheres would not be dissolved during embedding. The microspheres were easily seen by phase and UV microscopy. The number of phagocytosed spheres per 100 microns of RPE was counted and evaluated. Care was taken to insure that the spheres counted were actually ingested by the RPE cells and not simply attached to the cells.

RESULTS

In Vivo

The number of phagosomes per 100 microns of RPE length are higher in the tuftsin-treated eyes than in either the BSS-injected or the uninjected control eyes

[b]Fluoresbrite fluorescent microspheres (Polysciences).

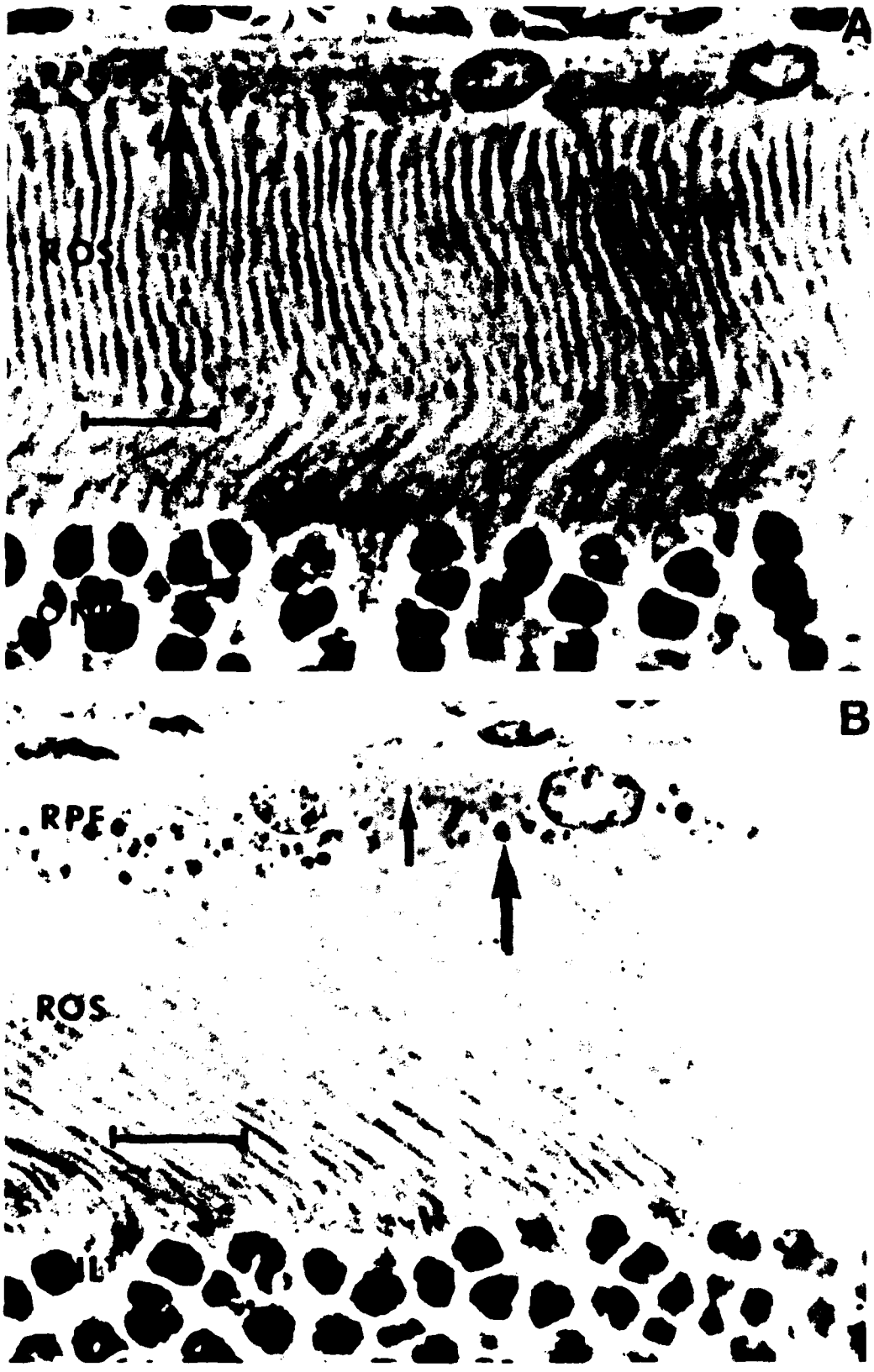

FIGURE 1. (A) Phase-contrast micrograph of outer retina from BSS-injected animal demonstrating few phagosomes (*arrow*) greater than one micron. Lee's stain. RPE-retinal pigment epithelium. ROS—rod outer segments. ONL—outer nuclear layer. Calibration bar on all micrographs = 10 micrometers.

(B) Phase-contrast micrograph from animal injected with 2 μl of 100 μg/ml tuftsin in BSS. Numerous large phagosomes are noted (*large arrow*) and are easily differentiated from mitochondria (*small arrow*). RPE—retinal pigment epithelium. ROS—rod outer segments. ONL—outer nuclear layer (± 1 S.E.M.).

(FIGURE 2). The number of phagosomes in the BSS-injected eyes is decreased from the untreated control counts, but the addition of tuftsin provided significantly greater phagocytosis than normal.

In Vitro

At seven hours of culture (six hours after adding spheres), the RPE was in excellent conditon (FIGURE 3). Apical processes had reformed and the cells appeared to be in good health. Latex spheres can be ingested by RPE,[17-19] and in the *in vitro* experiment were seen in both treated and untreated RPE. However, the number of spheres ingested by the tuftsin-treated cultures was significantly greater than those ingested by the untreated control cultures (FIGURE 4). Since no evidence was seen of digestion of spheres or of ejection of spheres from the basal aspect of the RPE, the number of spheres ingested represents a summation of all spheres phagocytosed over the six-hour incubation.

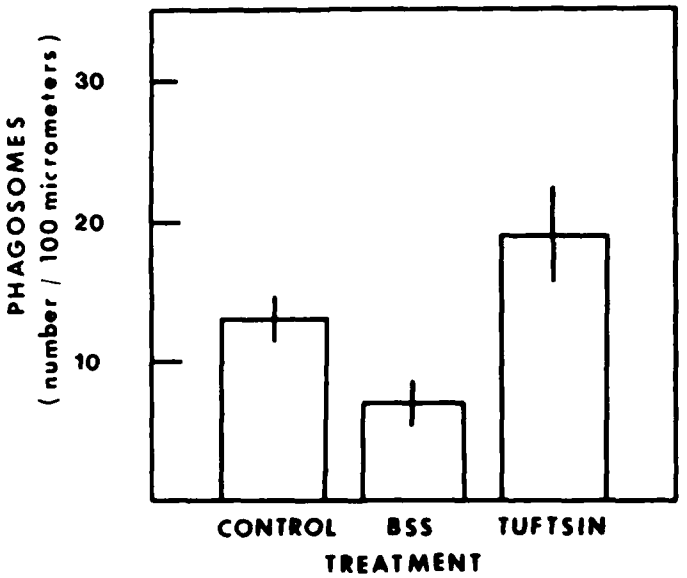

FIGURE 2. Number of phagosomes in control eyes, BSS-injected and tuftsin-treated, at 1 hour after lights on.

DISCUSSION

The results of the *in vivo* and the *in vitro* experiments described above lead to the conclusion that the RPE, like the macrophage, can be stimulated to phagocytosis by tuftsin. The actions of tuftsin are not confined to the derivatives of the embryonic mesoderm.

Four possibilities could, alone or in combination, explain the results of the *in vivo* experiments:

1. The RPE could have been directly stimulated as hypothesized.
2. Tuftsin could have caused an increased shedding of ROS that were phagocytosed by the RPE. If this were true, the increased number of phagosomes would be due to increased availability of the shed ROS packets.

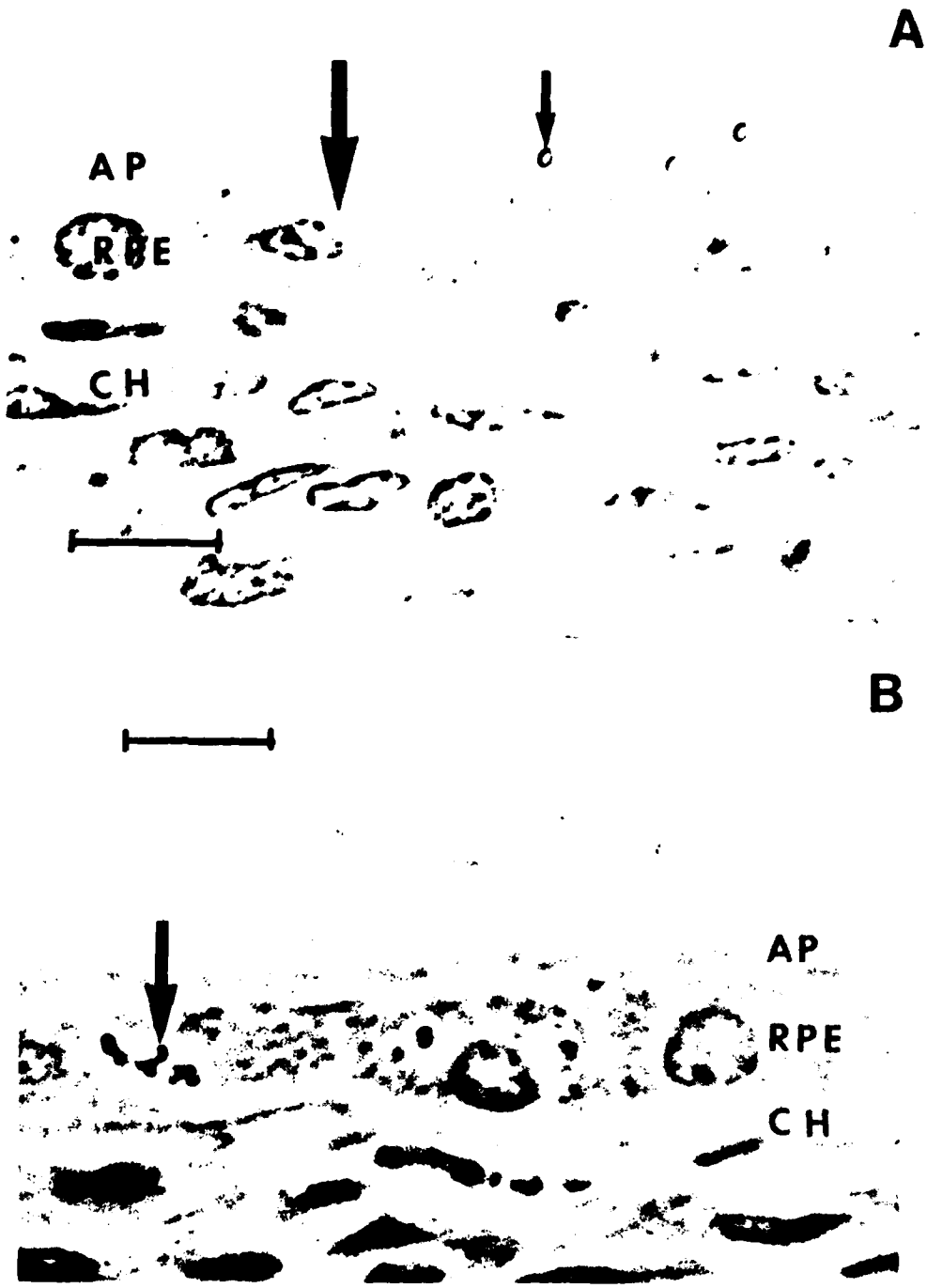

FIGURE 3. (A) Phase-contrast micrograph of cultured nontuftsin-treated RPE showing both ingested (*large arrow*) and attached (*small arrow*) fluorescent spheres. AP—apical processes. RPE—retinal pigment epithelium. CH—choroid.

(B) Phase-contrast micrograph of tuftsin-treated RPE. Numerous intracellular spheres are seen (*large arrow*). AP—apical processes. RPE—retinal pigment epithelium. CH—choroid.

3. Tuftsin might have impaired the ability of the RPE to degrade the phagosomes. In this case, the increased number counted would be due to an accumulation of undegraded phagosomes rather than the lower number of phagosomes usually seen.

4. Tuftsin could act as an opsonizing agent for the shed discs.

The results of the *in vitro* experiments argue against increased shedding or decreased degradation being the only factors responsible for the results observed *in vivo*. In the *in vitro* experiments, the spheres were available in equal quantities to both tuftsin-treated and control tissue. The greater number in the treated tissue demonstrated differential uptake rather than a differing availability of particles.

Latex spheres are not degraded by the RPE. If the increase in phagosomes seen in the *in vivo* experiment was due to an alteration of the degradative processes of the RPE, the spheres ingested by both treated and control cultures should be equal in number. Therefore, the difference between the number of ingested spheres in tuftsin-treated and control cultures must be due to a differential uptake of the microspheres.

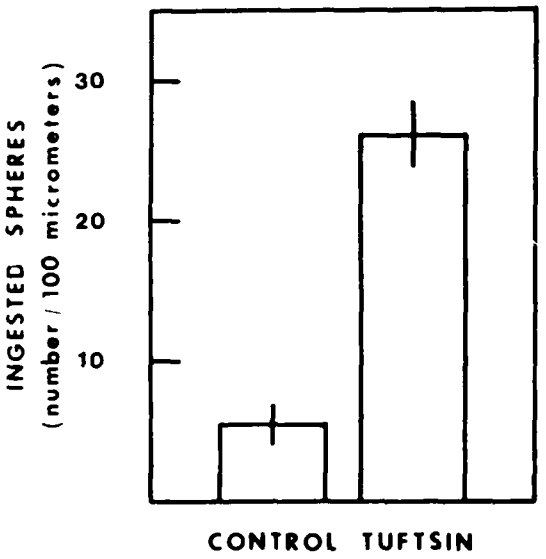

FIGURE 4. Number of ingested spheres counted in RPE that has been incubated in control medium or tuftsin-containing medium (± 1 S.E.M.)

The only difference between these sets of cultured tissues was the presence or absence of tuftsin. Other experiments in our laboratory indicate that tuftsin does not act as an opsonizing agent for particles exposed to mouse macrophages.[20] By extension of these results, the same should be true of the latex spheres or of the shed ROS discs. This could be directly examined by exposing the culture to tuftsin, washing, and then adding microspheres; or by bathing the microspheres in tuftsin, washing, and exposing them to phagocytosis by an untreated RPE culture. These experiments have yet to be done.

We conclude that the evidence from these experiments strongly supports the hypothesis that tuftsin directly stimulates phagocytosis by the RPE. The results also suggest that the phagocytic properties of the RPE, a tissue derived from the embryonic neuroectoderm, are similar to those of macrophages and other tissues derived from the embryonic mesoderm. If these properties are shown to be essentially the same, then the study of the interaction of the RPE and retina in health and disease will be greatly advanced by the body of knowledge already accumulated on the phagocytic mechanisms of macrophages. All of these considerations point to an exciting period of

research into the phagocytic properties of the RPE and the possibly vital role of tuftsin.

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PART V. ANTINEOPLASTIC EFFECTS OF TUFTSIN

In Vivo and *in Vitro* Antitumor Activities of Tuftsin[a]

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INTRODUCTION

It has been amply shown that the immunological system in the host plays several important roles in combating the occurrence of neoplasms and in protecting against bacterial infections. Although much emphasis in tumor immunology has been placed on studies of lymphocyte functions, substantial data have been accumulated that suggest that macrophages, natural killer (NK) cells, and polymorphonuclear leukocytes (PMN) are also likely to play equally important roles *in vivo*. Furthermore, it has been suggested that macrophages and NK cells impose the predominant lethal effect on tumor cells.[1,2] It has also been shown that PMN can exert a cytotoxic effect against tumor cells.[3] In addition, macrophages, PMN, and possibly NK cells[1-3] are capable of exerting antineoplastic effects through antibody-dependent cellular cytotoxicity (ADCC). Recently, a great deal of interest has centered around various agents that have been shown to activate macrophages and NK cells in a nonspecific manner. These include endotoxin, lipid A, double-stranded RNA, BCG, *Corynebacterium parvum*, levamisole, glucan, polyanions, OK 432, and interferons.[4,5]

An enormous number of factors or mediators are undoubtedly involved in the regulation of these immunological systems. Yet very few of these immunological mediators are chemically well defined. Tuftsin is one of a very few factors whose chemical identification has been convincingly established.[6]

By 1972, it was well established that tuftsin activated the phagocytic activities of PMN and macrophages. Based on this information, we wondered if tuftsin could also activate tumor cytotoxicity by these effector cells. If so, a role for tuftsin in the immunotherapy of neoplasia would be indicated. In order to test this question, we initiated animal studies in 1976 to examine the possible *in vivo* antitumor activity of tuftsin.

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IN VIVO STUDIES

We started our *in vivo* studies with the L1210 leukemia systems.[7] As shown in FIGURE 1, the first series of experiments showed clear-cut antitumor activity by tuftsin. The simultaneous injection of tuftsin with L1210 cells especially showed a drastic effect. The reduced effect seen in the animals receiving three injections at 24, 48, and 72 hr after the inoculation of L1210 cells, may indicate the importance of activating the host effector cells sufficiently before the leukemia cells started to proliferate. As we repeated this experiment, we made two crucial observations. First, L1210 cell lines obtained from different sources gave very different survival curves. This may be due to the difference in antigenicity in different cell lines. The second observation was that tuftsin preparations from different sources produced vastly different effects. In order to further examine antitumor activity of tuftsin *in vivo*, the Cloudman S-91 murine

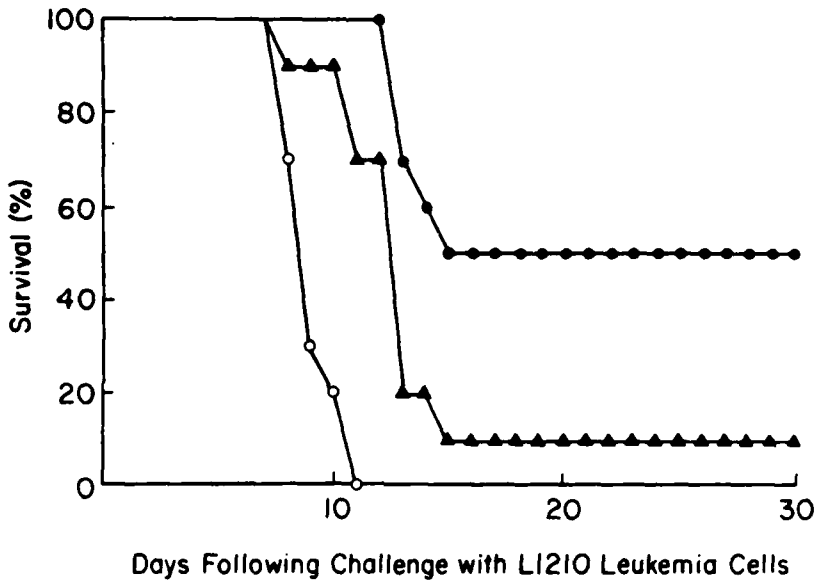

FIGURE 1. The effects of tuftsin on survival curves of DBA/2 mice challenged with L1210 leukemia cells. DBA/2 mice were divided into three groups of 10 mice. Control mice (—○—) were transplanted intraperitoneally (i.p.) with 0.3 ml of sterile saline containing 1×10^5 L1210 leukemia cells on day 0. One group (—●—) received an additional 0.2 μg of tuftsin i.p. simultaneously. The last group (—▲—) was treated with tuftsin (0.2 μg each) on days 1, 2, and 3.

melanoma was employed. Experimental mice (DBA/2) were injected subcutaneously (s.c.) with 2.5×10^5 tissue-cultured melanoma cells (CCL53-1) plus 10 μg tuftsin in 0.1 ml in the hind leg. Control mice received the tumor cells alone. The growth of melanoma was monitored for 48 days by measuring the sizes of progressively growing tumors. The melanomas in the tuftsin-treated group grew slower than those in the control group.[8] In order to assess the antitumor potential of tuftsin in a more quantitative manner, melanoma lung colonies were established by injecting cell-cultured Cloudman S-91 murine melanoma cells into the tail vein of DBA/2 mice.[9,10] In melanoma-injected mice, no tumor colonies were usually seen in the lungs during the first three weeks postinoculation. In subsequent experiments with this system to examine the antitumor effect of tuftsin, mice were sacrificed on day 31 after tumor implantation and pigmented melanoma colonies were counted following pneumonecto-

my. The experimental group of mice received 1×10^5 melanoma cells on day 0, and then were treated with i.p. injections of tuftsin three times per week continuously from day one until the termination of the experiment. As shown in TABLE 1, tuftsin-treated mice clearly displayed a significant reduction in melanoma lung colony formation. Mice treated with tuftsin two weeks after the inoculation of melanoma cells still also exhibited an antitumor effect.[11,12]

In order to confirm this antitumor activity of tuftsin, we decided to employ two primary tumor systems. The first system used was the Rous sarcoma model. We have investigated one strain of mice, C57BL/10Sn, which has a 65% incidence of Rous sarcomas.[13] Newborns received the oncogenic preparation in the left thigh within 24 hr of birth. We began single as well as multiple treatments of tuftsin, 10 or 25 μg per mouse, either one day or one week after the newborn mice received the oncogenic preparation. At the end of the 150-day observation period, mice receiving 25 μg of tuftsin one week following the oncogenic treatment, three times per week for either four or six weeks, developed significantly fewer tumors (44 and 30%, respectively). Mice treated in a similar manner beginning day one for four weeks showed a tumor incidence of 25%. The second system studied was 3-methylcholanthrene-induced primary fibrosarcoma.[14] Following the injection of 250 μg of the carcinogen in the left thigh, groups of 30 adult C57BL/10 mice were either treated with 10 μg of tuftsin i.p. three times per week or were untreated. At 130 days postinjection of 3-methylcholanthrene, significantly different tumor incidences of 53% and 83% ($p < 0.025$) were observed in the experimental and control groups, respectively. Although the tumor incidence in the treated group eventually increased during the 250-day observation period, the maximum tumor incidence (90%) in the control group was reached in only 151 days whereas 181 days were required to obtain a modestly lower tumor incidence (83%) in the experimental animals. The mean tumor latent period of 119 days for the tuftsin-treated group was significantly longer ($p < 0.0125$) than that of 103 days for mice in the untreated group.

In order to examine the antimetastatic activity of tuftsin in addition to its antitumor activity, we selected two more murine tumor systems. In the CH1 B-cell lymphoma model,[15] syngeneic male mice (B10-2^a4^bp/Wts) were given 1×10^6 CH1 cells intravenously (i.v.) followed one day later by various dosages of tuftsin given i.v. every other day.[16] As shown in TABLE 2, treatment with 0.001 or 0.01 μg per dose had no effect while a minimal effect was observed in mice receiving 0.1 μg. Mice receiving 1, 10, or 100 μg of tuftsin survived significantly longer than control animals. It is particularly noteworthy that the majority of mice in the groups receiving 10 or 100 μg of tuftsin showed no evidence of tumor growth at 150 days post tumor inoculation. The female mice showed an essentially similar result. We also examined the effect of tuftsin on the appearance of the s.c. nodules that appear following i.v. inoculation of CH1 cells into 2^a4^b mice. Mice receiving from 0.001 to 0.1 μg tuftsin per dose developed s.c.

TABLE 1. Effect of Tuftsin on the Formation of Melanoma Colonies in the Lung

Number of DBA Mice	Number of Melanoma Colonies per Mouse	Dosages of Tuftsin (μg/mouse)	$p <$
8	44.5 ± 15.9	—	—
8	2.6 ± 2.5	1,000	0.0005
8	17.5 ± 17.1	100	0.005
8	4.9 ± 4.2	10	0.0005

TABLE 2. Effect of Tuftsin on CH1 Tumor-bearing Mice[a]

Group	Sex	Tuftsin[b] (μg/dose)	Survival (days)	Mean Survival	Survival Total
A	M	None	30, 32, 33, 36, 36, 37, 37	34.4	0/7
B	M	0.001	26, 31, 32, 35, 35, 37, 37	33.3	0/7
C	M	0.01	32, 32, 32, 33, 36, 36, 37, 38	34.5	0/8
D	M	0.1	31, 31, 33, 35, 35, 35, 38, 41	34.9	0/8
E	M	1.0	32, 33, 37, 37, 39, 39, 39, 47	37.9	0/8
F	M	10	43, 44, 45, 5 mice > 150	—	5/8
G	M	100	45, 47, 6 mice > 150	—	6/8
H	F	None	29, 31, 31, 31, 32, 33, 37	32.0	0/7
I	F	1.0	36, 36, 38, 39, 40, 40, 43, >150	—	1/8
J	F	10	36, 37, 38, 5 mice > 150	—	5/8
K	F	100	38, 43, 45, 45, 41, 3 mice > 150	—	3/8

[a]All mice received 1×10^6 CH1 cells i.v. on day 0.
[b]Tuftsin-treated animals received tuftsin every other day i.v. beginning on day 1.

tumors in a manner similar to the control animals. Animals receiving 1 μg per dose displayed a reduced number of s.c. tumors while no tumors were observed in mice receiving 10 or 100 μg of tuftsin. Daily injections of tuftsin did not improve the results. When similar experiments were performed with animals receiving tuftsin i.p., very little therapeutic effect was observed suggesting that, at least in this model, the i.v. route of administration is more effective. A preliminary experiment was also performed to determine the cellular mechanism by which tuftsin exerts its antitumor activity. Mice receiving CH1 tumor cells were either left untreated, received tuftsin every other day, received carrageenan every other day, or received carrageenan alternating with tuftsin. Carrageenan given in combination with tuftsin reduced the survival to control levels. Since carrageenan has been shown to suppress both macrophages[17] and NK activity,[18] the reversal of the immunopotentiating effects of tuftsin by this compound suggest one or both of these cell types are probably responsible for the antitumor activity observed. In addition, macrophages and NK cells appear to play a role in controlling the growth of CH1 cells in mice not treated with tuftsin as carrageenan treatment by itself reduced the survival time of the mice compared with control animals. We have also initiated a Lewis lung carcinoma study,[19] as it metastasizes to the lung following s.c. implantation and therefore serves as a very good model for assessing the effectiveness of tuftsin as an inhibitor of metastatic tumor development. Our preliminary experiment indicates a positive effect of tuftsin. We are currently initiating applications of liposomes[20] to these animal systems. As described above, our *in vivo* studies employing a variety of murine tumor models, clearly established the antitumor activity of tuftsin *in vivo*.

IN VITRO STUDIES

In order to elucidate the cellular mechanism behind the ability of tuftsin to inhibit growth of tumors, we have performed various *in vitro* experiments. To rule out the possibility that tuftsin possesses direct antitumor activity or is cytotoxic to tumor cells, the effect of tuftsin on the incorporation of [^{3}H]-thymidine by target cells, L1210 leukemia cells[7] and Cloudman S-91 melanoma cells,[10] was examined. No direct effect

of tuftsin was observed. Therefore, the antitumor activity of tuftsin appeared to be exerted through the activation of immunological effector cells. The effect of tuftsin on the cytotoxicity of peritoneal macrophages from DBA/2 mice against cultured L1210 leukemia cells was then examined.[7] Monolayered peritoneal macrophages were treated with 1 or 10 μg per ml of tuftsin for 18 hr in microtiter plates. After washing, L1210 leukemia cells labeled with [^{3}H]-proline were introduced to each well at an effector:target cell ratio of 50:1. After 48 to 72 hr of incubation, the cytotoxicity was determined. The tuftsin treatment resulted in significantly elevated macrophage cytotoxicity.

As our *in vivo* experiment showed reduced melanoma colony formation in the lungs, cytotoxicity of alveolar macrophages was also studied since they appear to represent the major class of effector cells in the lungs. Alveolar macrophages were collected from DBA/2 mice by gentle endobronchial lavage to prevent the introduction of blood and with the aid of lidocaine. These effector cells were then treated with tuftsin for one hour and ^{51}Cr-labeled Cloudman S-91 melanoma cells introduced. The standard 18-hr, ^{51}Cr-release assay[21] clearly displayed the enhanced cytotoxicity by tuftsin treatment while the untreated effector cells showed no significant cytotoxicity. We have also observed the activation of alveolar macrophage *in vivo* by tuftsin administration to DBA/2 mice.[12] Mice received i.v. doses of tuftsin ranging from 1 μg to 500 μg per animal. Alveolar macrophages were collected from animals 24 to 120 hr after receiving tuftsin, and their cytotoxicities were measured against Cloudman S-91 melanoma cells using the ^{51}Cr-release assay. No cytotoxicity was observed with macrophages collected from mice treated with 1 or 10 μg of tuftsin. Alveolar macrophages from mice receiving 100 μg were completely inactive at 24 hr, very cytotoxic at 48 hr, slightly cytotoxic at 72 hr, and unreactive at 96 and 120 hr. The effector cells from mice treated with 500 μg of tuftsin were slightly cytotoxic at 24 hr, reached peak cytotoxicity at 48 hr, remained cytotoxic at 72 and 96 hr, and were unreactive at 120 hr. We have also examined the plastic-adherent cells, presumably the Kupffer cells, from the liver of the mouse and found that they can be activated with tuftsin.

The effect of tuftsin on granulocyte cytotoxicity was also examined using [^{3}H]-proline-labeled target cells.[22] Granulocytes from human blood were purified, treated with tuftsin, and tested against established human malignant melanoma cell lines, and a normal human embryonic lung cell line, WI-38. Granulocytes showed rather weak but significant cytotoxicity against human melanoma cell lines, but failed to show significant cytotoxicity against the normal cell line WI-38. Although untreated peritoneal macrophages always showed substantial natural cytotoxicity against tumor cells in our study,[7] granulocytes as well as murine alveolar macrophages[12] did not exhibit significant cytotoxicity without tuftsin treatment. Microscopic examination of the microtiter wells showed no phagocytosis of tumor cells by the granulocytes indicating that the cytotoxicity of granulocytes was not expressed by enhanced phagocytosis.

With regard to the effect of tuftsin on NK cells, an extensive study has been carried out using crude and purified mouse[21] and human NK cells in our laboratory. This investigation was further enhanced by the observation of specific tuftsin receptor on NK cells. We have also examined natural cell-mediated cytotoxicity using human peripheral leukocytes as effectors and two human cell lines, K-562 leukemia cells and 39.5 human melanoma cells, as target cells. Human peripheral white blood cells were found to express augmented natural cell-mediated cytotoxicity when treated with tuftsin. Tuftsin-treated effector cells displayed significantly enhanced natural cell-mediated cytotoxicity against 39.5 and K-562, respectively. Preliminary cell fractionation studies indicated that NK cells, macrophages, and granulocytes all displayed

enhanced natural cell-mediated cytotoxicity when treated with tuftsin *in vitro*.[23] This result again supports our observation indicating the presence of tuftsin receptors on granulocytes, macrophages, and NK cells.

OTHER RELEVANT STUDIES AND DISCUSSION

In the preceding sections, we summarized our studies dealing with *in vivo* and *in vitro* antitumor activities of tuftsin. There have been a number of *in vivo* studies published. Bruley-Rossett *et al.*[24] have reported on a drastically reduced incidence of spontaneous tumors in aged mice by injecting tuftsin weekly. They have also observed the restoration of the aging-related impaired cytotoxic capacities of macrophages and cytolytic T cells of mice by repeated administration of tuftsin. Since we failed to observe the direct binding of tuftsin to purified T cells, we feel that the activation of T cells was brought about indirectly through the activation of macrophages. Employing synthetic tuftsinyltuftsin, Najjar *et al.*[25] have demonstrated an *in vivo* antitumor effect in the L1210 leukemia model. Catane *et al.*[26] have also shown the antitumor activity of this peptide against 3-methylcholanthrene-induced transplantable fibrosarcomas in C3H mice.

TABLE 3. Effect of Tuftsin on Mouse Peritoneal Cells[a]

	Saline Control		Kenstin Thr-Pro-Arg-Lys		Tuftsin Thr-Lys-Pro-Arg	
Days after Injection	Dark Cell (%)	Total Cell	Dark Cell (%)	Total Cell	Dark Cell (%)	Total Cell
4	35	1.5×10^7	33	1.3×10^7	39	1.4×10^7
7	49	1.5×10^7	50	1.4×10^7	72	2.5×10^7

[a]CBA mice were injected with one ml of sterile saline containing 10 μg peptide. After four and seven days, the peritoneal exudate collected from two mice were examined as described.[7]

There have been several reports on *in vitro* antitumor effect of tuftsin. Florentin *et al.*[27] showed activation of the cytostatic effect of macrophages and enhancement of ADCC activity in the spleen cells in tuftsin-treated mice. We have also examined the effect of tuftsin on ADCC using a similar system except that spleen effector cells were treated directly with tuftsin *in vitro*. Thus splenic effector cells were incubated with various concentrations of tuftsin (1 to 100 μg per 0.2 ml) for one hr, tuftsin was then washed off and the ADCC activity was assayed employing ^{51}Cr-labeled chicken cells coated with rabbit antibody. A twofold rise in cytotoxicity was observed with cells incubated with 50 μg of tuftsin while 5, 10, and 100 μg gave significant but less dramatic increases in cytotoxicity.

There are other known properties of tuftsin that may help tuftsin express its antitumor activity *in vivo*. We and others showed that tuftsin enhanced random migration of leukocytes and was chemotactic.[6,8,28-33] These properties of tuftsin may help mobilize effector cells to the tumor site. In our study, while i.p. injection of tuftsin increased peritoneal leukocyte counts in mice, kentsin, which has the same amino acid composition as tuftsin but a different sequence, showed no such effect (TABLE 3). In these harvested macrophages, we found higher percentages of dark phase cells or activated macrophages in the tuftsin-treated group than in the controls.[7] Tzehoval *et*

al.[34] showed that tuftsin augmented the antigen-specific, macrophage-dependent education of T lymphocytes. Tuftsin was also shown to potentiate the antibody response to a thymus-dependent antigen.[27] With the augmented antibody formation, tuftsin may possibly enhance ADCC.

We also examined the ability of tuftsin to stimulate proliferation of human peripheral leukocytes and mouse spleen cells by studying the effect of tuftsin on the incorporation of [^{3}H]-thymidine into these cell types.[35] Tuftsin appeared to be mitogenic but weakly so when compared with the standard lectins such as PHA, Con A, and LPS. Tuftsin was mitogenic for both C57BL/10Sn and DBA/2 spleen cells at a concentration of 0.1 to 50 μg per culture (0.2 ml). The optimal concentration of tuftsin that gave a three- to fourfold stimulation was between 1.0 and 10 μg per culture at 24 or 48 hr. The maximal stimulatory effect (3-fold) of tuftsin on human mononuclear cells was found after 72 hr at a concentration of 1 μg per culture. Studies to determine the cell populations activated by tuftsin are now under way. This function of tuftsin may contribute to the antitumor activity by increasing numbers of available effector cells. Our study also indicates that tuftsin possess hemopoietic colony-forming activity. This property may also augment the antitumor activity of tuftsin *in vivo* by enhancing differentiation and maturation of the effector cells.

Although the direct cytotoxicities of macrophages, granulocytes, and NK cells were extensively studied in our investigation of tuftsin, indirect stimulation of other effector cells through the release of various factors from these tuftsin-activated cells may play an important role in the *in vivo* antitumor effect of tuftsin. As mentioned, the activation of cytolytic T cells may be achieved in this manner.

The aforementioned experimental studies and discussion strongly indicate that tuftsin immunotherapy may prove effective under various pathological conditions associated with neoplasia. Consequently, the frequent presence of life-threatening infections among cancer patients, the availability of a sensitive immunoassay[36] that permits measurement of serum tuftsin levels at any given time, as well as the low toxicity of tuftsin[26] make it one of the most promising natural biological response modifiers for the clinical treatment of neoplastic diseases.

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Immunorestorative Capacity of Tuftsin after Long-Term Administration to Aging Mice

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INTRODUCTION

Many attempts have been made to restore the depressed immune functions of old animals. Newborn thymus and adult bone marrow have been shown to reconstitute the antibody and mitogenic response of long-lived hybrid mice for long periods of time.[1] However, results obtained with thymic extracts have been less encouraging, leading to limited and transient stimulation of the immune functions of aged mice;[2,3] nevertheless, these results support the fact that primary age-related changes involve mainly regulatory T-cell reactions. Immunostimulating drugs such as naturally occurring or synthetic compounds have been utilized infrequently. In our hands, bestatin, an antibiotic, was able to restore some T-cell functions and to increase the life expectancy of treated animals.[4] We reported in the same study that the incidence of spontaneous tumors arising in old mice is significantly reduced following bestatin administration.

Many studies have established that tuftsin, a physiological tetrapeptide, preferentially stimulates macrophage functions and that it has significant tumoricidal activity.[5-7] The present work was designed to see whether tuftsin given over a long period of time would display an inhibitory effect on spontaneous development of tumors associated with aging in C57BL6 mice, and in that case what cellular immune antitumor mechanisms could be implicated. Results indicate that in this experimental model, tuftsin exhibits an antitumor activity associated with an increase in the cytostatic capacity of macrophages as well as in cytolytic T-cell activity.

MATERIALS AND METHODS

Mice

Inbred C57BL/6 female retired breeders were purchased at the age of 6 months from BOM Laboratories (Ry, Denmark); they were kept in conventional housing before treatment and during the time of the treatment, that is, from the age of 6 months to 19 months. Controls were 2-month-old C57BL/6 females.

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Drug and Treatment

Tuftsin, a synthetic polypeptide (threonyl-lysyl-prolyl-arginine), was prepared by J. Martinez (Montpellier, France). This drug was administered in a dose of 10 μg/mouse by weekly i.p. injections to 12- to 18-month-old mice. Thirty mice were included in the saline-treated (controls) and tuftsin-treated groups at the beginning of the study, but only 27 in the control and 22 in the tuftsin groups were evaluated. All the animals were inspected twice a week, or more often when they were thought to be ill. A routine postmortem examination was performed on mice that either died naturally or were killed because of infection or suspicion of tumor. Abnormal tissues were prepared for microscopic examination. The histologic types of the tumors were determined and the percentage of tumor-bearing animals was evaluated.

After six months of treatment, the mice were tested for the following immunological functions:

Immunologic Assay for Macrophage-mediated Cytostasis

The *in vitro* cytostatic activity for peritoneal macrophages was determined by the [^{3}H]-TdR-incorporation test[4] in which 5×10^4 P815 tumor cells/well were cultivated on macrophages monolayers at an effector-to-target ratio (E:T)of 10 macrophages:1 tumor cell. After 18 hours of incubation at 37°C, cultures were pulsed with 1 μCi [^{3}H]-TdR (20 Ci/mmol). [^{3}H]-TdR incorporated into tumor cells was measured in a beta counter. Results are expressed as mean counts per minute of [^{3}H]-TdR incorporation ± standard error of six cultures and as percent inhibition of tumor cell proliferation.

Immunologic Test for Natural Killer (NK) Cell Activity

Various numbers of spleen cells were incubated for 4 hours at 37°C with 10^4 ^{51}Cr-labeled YAC-1 lymphoma cells in U-well microtest plates. The amount of ^{51}Cr released in the supernatants was measured in a gamma counter. The percentage of specific lysis was calculated as follows: percent lysis = [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100.

Antibody-dependent Cell-mediated Cytotoxicity (K Cell)

Various numbers of mouse spleen cells were incubated for 18 hours at 37°C with 10^4 ^{51}Cr-labeled CRBC in U-well microtest plates in the presence of a 1:20,000 final dilution of rabbit anti-CRBC serum. The amount of ^{51}Cr released in the supernatants was measured in a gamma counter. The percentage of specific lysis was calculated as for NK-cell activity, but spontaneous release was determined from the mean lysis in the cultures containing target and effector cells but no antiserum.

Cytolytic T-Cell Activity

For 4 days, 5×10^7 spleen cells were incubated at 37°C in falcon flasks in the presence or absence of mitomycin C-treated P815 tumor cells. The RPMI 1640 culture

TABLE 1. Peritoneal Macrophage Cytostatic Activity against Tumor Cells: Effect of Age and Tuftsin

		Macrophages from:		
	No Macrophages	Untreated 2-Month-Old Mice	Untreated 18-Month-Old Mice	Tuftsin-treated 18-Month-Old Mice
[^{3}H]-TdR incorporation into tumor cells (in CPM ± SE)	30,300 ± 2,542	8,660 ± 685	28,664 ± 1,041	10,698 ± 810
% Inhibition[a] of tumor cell proliferation	—	71.5%	5.4%	64.7%

[a] $\%\ \text{Inhibition} = 100 - \left[\dfrac{\text{mean number of [}^3\text{H]-TdR (CPM) incorporated into tumor cells in the presence of macrophages}}{\text{mean number of [}^3\text{H]-TdR (CPM) incorporated into tumor cells alone}} \times 100\right]$

medium contained antibiotics and glutamine with or without addition of 5×10^{-5} *M* 2-mercaptoethanol (2-Me). After 4 days of culture, the suspensions were centrifuged and the number of viable cells was enumerated. Various numbers of spleen cells were incubated in U-well microtest plates for 4 hours at 37°C in the presence of 10^4 ^{51}Cr-labeled P815 tumor cells. The amount of ^{51}Cr released in the supernatants was measured in a gamma counter. The percentage of specific lysis was calculated as for NK activity, but spontaneous release was obtained by means of lysis of cultures containing 10^4 ^{51}Cr-labeled P815 as targets and various numbers of spleen cells cultivated in the absence of P815 tumor cells.

RESULTS

After 6 months of treatment, the immunologic reactivity of the mice given tuftsin was compared to that of controls.

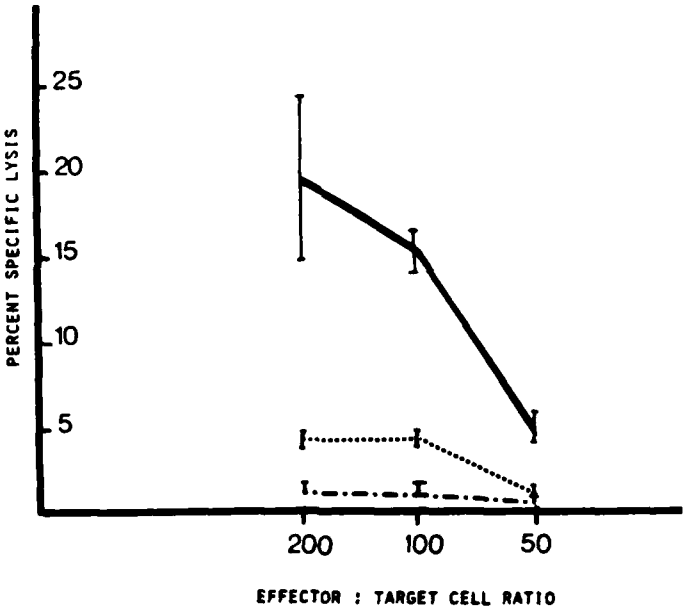

FIGURE 1. NK activity of spleen cells from untreated young mice (————), untreated aged mice (----), or aged mice treated with tuftsin (— — —).

Peritoneal Macrophage Cytostatic Activity

The effect of the treatment on the capacity of macrophages to inhibit tumor cell proliferation was measured by the inhibition of [^{3}H]-TdR incorporation. Tumor cells were cultivated in the presence of macrophages originating from treated or untreated animals, and the results are presented in TABLE 1. The decreased cytostatic capacity of macrophages associated with aging (5.4% inhibition) was restored by treatment with tuftsin (64.7% inhibition), resulting in levels of macrophage activity observed in young mice (71.5% inhibition).

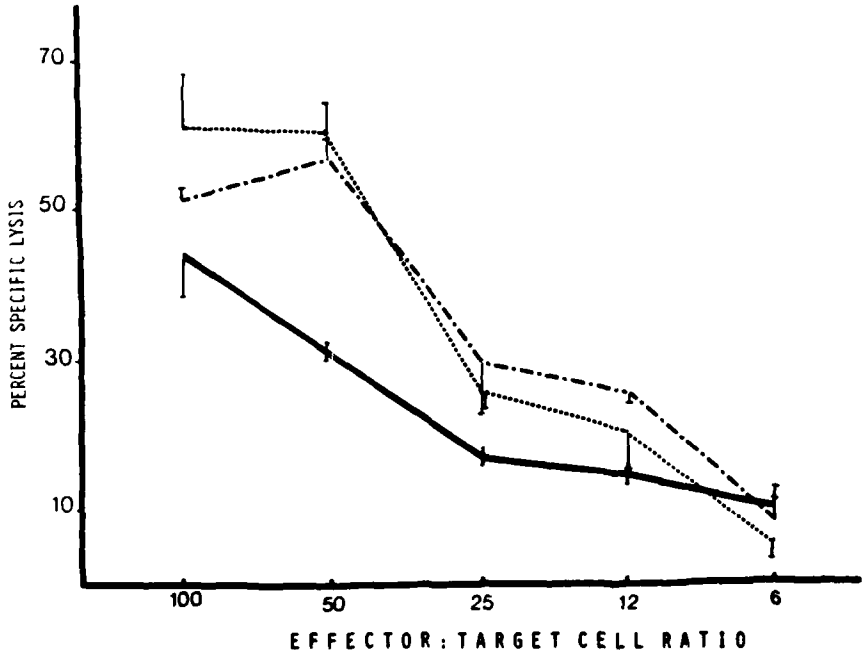

FIGURE 2. ADCC of spleen cells from untreated young mice (————), untreated aged mice (----), or aged mice treated with tuftsin (— - —).

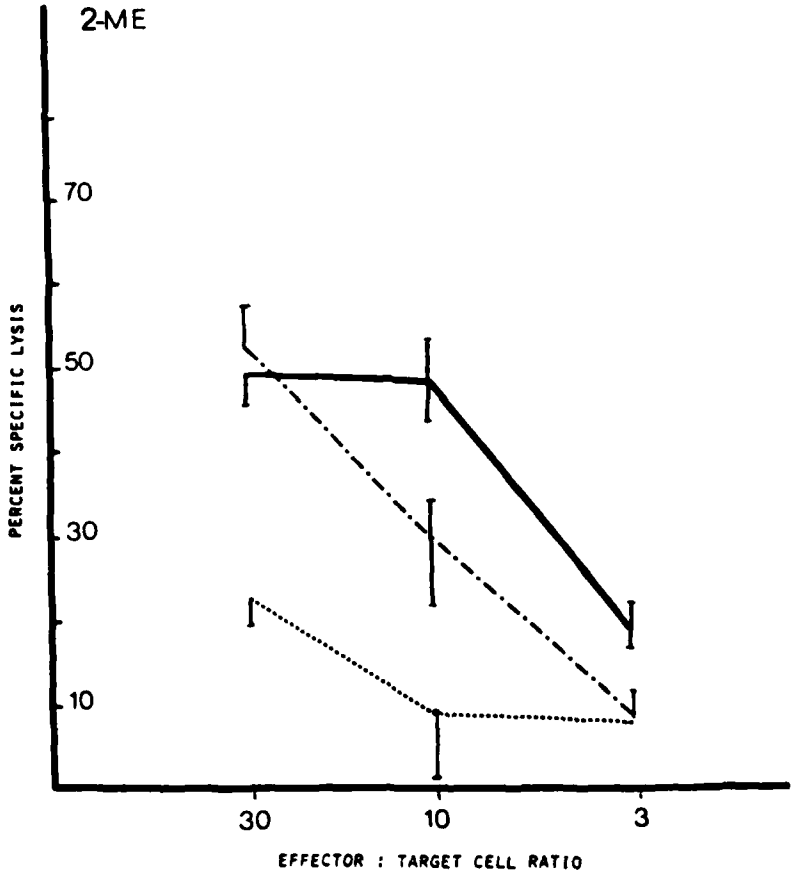

FIGURE 3. Cytolytic T-cell activity for P815 tumor cells of spleen cells from untreated young mice (————), untreated aged mice (----), or aged mice treated with tuftsin (— - —) after 4-day *in vitro* allogeneic stimulation by P815 tumor cells in the presence of 5×10^{-5} 2-Me in culture medium.

Natural Killer Cell Activity

In our experiment, age considerably reduced the NK-cell activity of splenic cells (E:T = 200:1) from 18% specific lysis for young mice to 4.7% for 18-month-old mice. NK-cell activity remained low after tuftsin treatment (FIG. 1).

Antibody-dependent Cell-mediated Cytotoxicity

As we have already observed in the previous work, K-cell activity for CRBC coated with rabbit anti-CRBC was elevated as the mice aged. Tuftsin treatment did not modify this activity (FIG. 2).

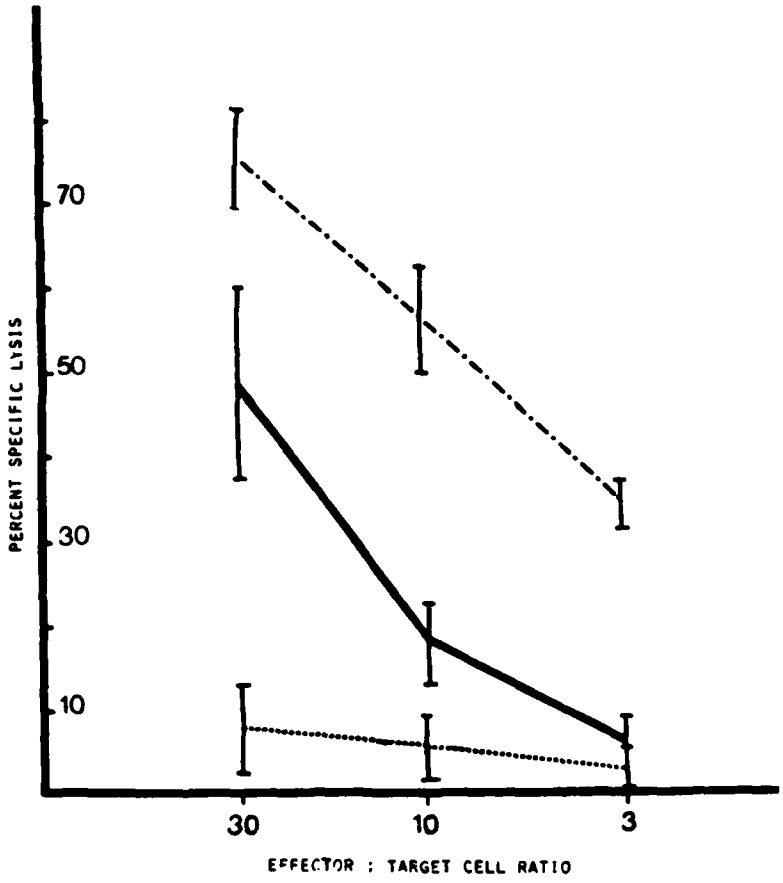

FIGURE 4. Same as FIGURE 3 in the absence of 5×10^{-5} 2-Me in culture medium.

Cytolytic T-Cell Activity

The capacity of spleen cells to generate cytolytic T cells after an *in vitro* allogeneic stimulation was tested by the use of two experimental protocols in which cells were derived from both treated and untreated groups of mice. The cultures were routinely incubated in the presence of 2-Me, which effectively enhanced nonspecific immunologic cellular activities *in vitro*.[8] However, Makinodan and Albright[9] have demonstrated that 2-Me can much more effectively enhance the *in vitro* response of splenic

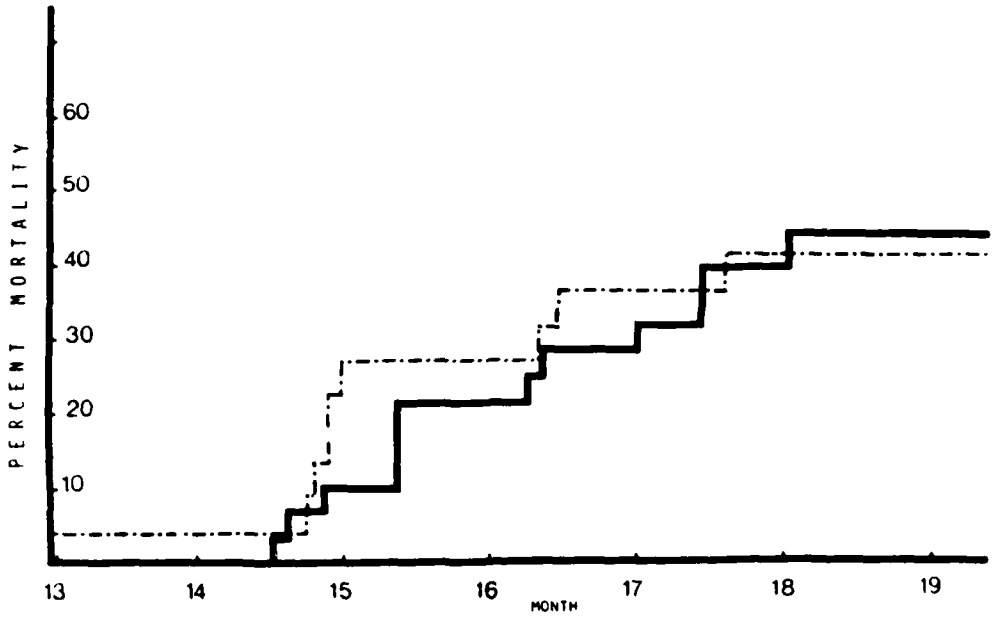

FIGURE 5. Percent mortality related to aging (by month) in mice either untreated (————) or treated with tuftsin (— — —).

cells derived from old animals than that of splenic cells derived from young animals. For this reason the formation of cytolytic T cells was studied both in the presence (FIG. 3) and in the absence (FIG. 4) of 2-Me. 2-Me enhanced the cytolytic activity of spleen cells from both young and old donors. Tuftsin stimulated cytolytic T cells in the presence of 2-Me, but in its absence, tuftsin-stimulated activity of aged mice surpassed that of young mice.

Mortality and Histologic Examination

The number of mice dead at 18 months of age was similar in tuftsin-treated group (9/22 = 40.9%) compared to the control group (12/27 = 44.4%) (FIG. 5). Histologic examination revealed that most of the mice dead during the course of the treatment have intercurrent diseases. All the tumors (except one in the control group) were detected when the animals were sacrificed for immunologic testing between 18 and 19 months. Of untreated aged mice, 6/27 (22%) were found to bear a malignant tumor (TABLE 2). In contrast, tuftsin treatment inhibited or only delayed tumor development (0/22).

TABLE 2. Incidence and Histologic Form of Spontaneous Tumors: Effect of Tuftsin

	Treatment Applied to B6 Mice From 12 to 16 Months of Age		
	Saline		Tuftsin (10 μg/mouse)
Number of Tumor-bearing Mice ÷ Total Number of Mice in Group (%)	6/27 (22%)	$p < 0.01$	0/22 (0%)
Histologic form of the tumors	2 Adenocarcinoma 2 undifferentiated malignant lymphoma 1 Immunoblastic lymphoma 1 Malignant lymphoma (unclassified)		—

DISCUSSION

The results of the study described here demonstrate a strong effect of tuftsin, given repeatedly, on the immunodepressed functions of 18-month-old B6 mice. Tumoricidal activity of peritoneal macrophages and *in vitro* generation of cytotoxic T-cell activity were increased to the levels observed in normal young mice. However, NK- and K-cell activities were not modified in aged control or tuftsin-treated mice. A significantly decreased percentage of tumor-bearing animals was found in the tuftsin-treated group as compared to untreated mice of the same age.

Stimulation of the tumoricidal function of the macrophages confirmed the published data showing that tuftsin acts mainly on phagocytic cells.[5,6,10] This increased capacity of macrophages to kill tumor cells may be, in our experimental system, one of the host's defense mechanisms effective in the control of malignant development as described in many other situations.[11]

In addition, a complete restoration of the cytotoxicity mediated by T cells was observed after tuftsin treatment. It is likely that such an effect did not result from a direct action of tuftsin on T cells but was rather the consequence of an increased macrophage-T cell interaction. Tzehoval[12] demonstrated that presentation and processing of antigen by macrophages improved by tuftsin was associated with a more effective immune response. Moreover, our observation[13] that antibody response to T-cell-dependent antigens was increased by tuftsin led to similar conclusions. However, although the cytolytic T-cell activity may represent a specific antitumor immune response,[14] it is not likely that this function plays any role in the surveillance of tumor development since spontaneous tumors are known to be nonimmunogenic or weakly so.

On another hand, the functional deficiency of spleen cells observed with aging could be associated with a reduced amount of tuftsin released in the serum as described in splenectomized animals.[15] This alternative hypothesis, which remains to be demonstrated, would give an explanation of the regulatory role of tuftsin given *in vivo* to aged mice.

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Toxicology and Antitumor Activity of Tuftsin[a]

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INTRODUCTION

Tuftsin is a naturally occurring basic tetrapeptide with the sequence Thr-Lys-Pro-Arg.[1] It is an integral component of immunoglobin G and is released from its parent molecule by enzymatic cleavage.[1,2] Tuftsin has been shown to stimulate phagocytosis by human and animal polymorphonuclear leukocytes[1,3] and macrophages.[3,4] Additional properties of tuftsin include enhancement of the migration response of human mononuclear cells[5] and augmentation of antigen-specific, macrophage-dependent sensitization of T lymphocytes.[6] When added *in vitro* to peritoneal macrophages, tuftsin conferred a high cytostatic activity against syngeneic tumor cells.[7] More recent experiments suggested that tuftsin has antitumor activity *in vivo* against murine tumors.[8,9]

In order to evaluate the potential of tuftsin as an agent for the treatment of human cancer, we conducted a series of experiments in animals. The lethal dose (LD_{50}) of tuftsin for mice was first determined and the biological effects of lower doses of tuftsin were then examined in normal mice and dogs.

In this paper, we will review our observations on the biological effects of tuftsin administered to animals, both normal mice and dogs, as well as tumor-bearing mice, and the results of a preliminary Phase-I clinical trial with tuftsin in cancer patients.

ACUTE LETHAL TOXICITY IN MICE

SNZ mice (weight range of 19–21 g) were injected intravenously (i.v.) with different doses of tuftsin. Mortality within 24 hours was monitored. We found that the acute LD_{50} in mice was 2.4 g/kg body weight (TABLE 1). Since tuftsin was administered in these experiments as a 10% water solution, the acute mortality of the mice may have resulted from the high volumes of the viscous tuftsin solution, injected intravenously.

[a]This work was supported by the National Council for Research and Development, Israel.

ACUTE TOXICITY STUDIES IN DOGS

Three beagle dogs, weighing 15, 18, and 11.5 kg, were injected i.v. with a solution of 1% tuftsin. The dogs were closely observed for any change in their behavior, and the respiration rate and pulse were measured every 10 minutes during the first hour after tuftsin injection. Blood samples were obtained before tuftsin administration and one hour, two days, one week, and two weeks after the administration of tuftsin. The blood samples were tested for the following parameters: complete blood count, serum electrolytes (including $Na+$, $K+$, HCO_3^-, Ca^{2+}, PO_4^-), BUN, creatinine, uric acid, glucose, albumin, globulin, bilirubin, cholesterol, alkaline phosphatase, transaminases, and LDH.

All the parameters tested did not show changes except for the counts of WBC. The pulse and respiration rates remained stable during the first hour after administration of tuftsin with no change as compared to preinjection rate. There was no change in the pattern of behavior of the dogs during three weeks of follow-up and their appetite remained normal. All biochemical parameters examined in the serum samples remained within normal limits throughout the period examined. However, there was a significant increase in the WBC counts in all dogs tested that appeared between two days and one week following tuftsin administration. During the same time period, the

TABLE 1. Mortality Data for Mice Receiving Different Doses of Tuftsin

Dosage (g/kg)	Mortality Ratio	
	Males	Females
1	0/2	0/2
2	0/7	0/7
2.4	2/5	3/5
3	5/5	4/5
3.75	5/5	5/5
4	2/2	2/2
6	2/2	2/2

erythrocyte or platelet counts remained within normal limits (TABLE 2). The leukocytosis persisted for up to two weeks, which was the maximal period of follow-up in this set of tests. It should be noted that the differential count was without significant change throughout this period, indicating that the increase in the total number of the leukocytes was common for the various subgroups of the peripheral blood white cells and did not result from an increase in the number of monocytes only (TABLE 2).

EFFECT OF TUFTSIN ON LEUKOCYTE COUNTS IN MICE

BALB/c mice at the age of either 2–3 months or 12–18 months (referred to hereafter as "young" or "old" mice, respectively), were injected either i.v. or interperitoneally (i.p.) with tuftsin, 0.01–25 mg/kg in 0.2 ml saline. Following different periods of time, 0.02 ml of blood were drawn from the medial canthus of the eye. This procedure was well tolerated by the mice without serious damage even after multiple bleedings. The total white blood cells (WBC) were counted in each blood sample. The results indicated that doses of 0.5 and 5.0 mg/kg of tuftsin consistently produced a leukocytosis in both groups of the injected mice. The maximal effect was

TABLE 2. Blood Counts and Differential in Dogs Following the i.v. Administration of 1 mg/kg Tuftsin

Time	Erythrocytes $\times 10^{12}/l$	Platelets $\times 10^{9}/l$	WBC $\times 10^{9}/l$	Polymorphonuclear Cells				
				Neutrophils %	Eosinophils %	Basophils %	Lymphocytes %	Monocytes %
Before tuftsin	7.4 ± 0.2	184 ± 41	5.9 ± 0.7	72.3 ± 2.3	3.3 ± 0.9	0	23.3 ± 2.0	1.0 ± 0.6
After one hour	7.7 ± 0.2	209 ± 26	6.1 ± 0.6	68.0 ± 1.5	2.7 ± 0.9	0	28.7 ± 2.9	2.7 ± 0.3
Two days	8.0 ± 0.3	238 ± 18	6.8 ± 0.4	64.3 ± 1.3	2.3 ± 0.7	0	29.7 ± 0.3	3.7 ± 1.5
Seven days	7.7 ± 0.1	228 ± 20	14.4 ± 1.6	64.0 ± 3.2	3.0 ± 2.1	0	26.7 ± 1.5	6.3 ± 2.9
Fourteen days	7.6 ± 0.3	195 ± 15	13.4 ± 1.5	72.7 ± 4.4	1.0 ± 0.6	0	23.3 ± 4.4	3.0 ± 0.6

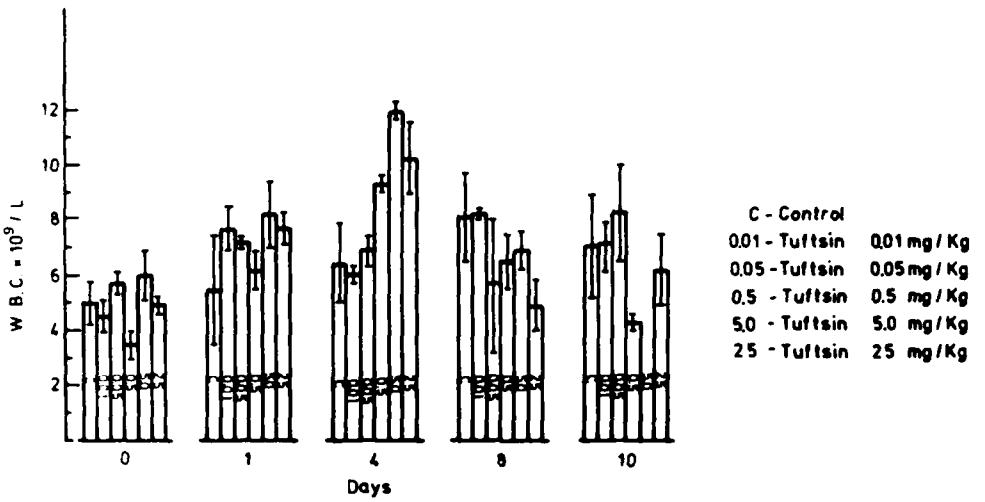

FIGURE 1. White blood cell counts in healthy BALB/c mice, 12–18 months old, following the intravenous administration of various doses of tuftsin. Control mice received buffered saline only.

observed after four days in the "old" mice. In the "young" mice, leukocytosis was already found on the first day, and the level of response to tuftsin was significantly higher (FIGS. 1 and 2). The tuftsin-induced leukocytosis lasted for approximately one week.

EFFECT OF TUFTSIN ON THE CYTOTOXIC ACTIVITY OF SPLEEN CELLS IN MICE

The cytotoxic activity of C3H/eb mouse spleen cells was tested in a 24-hour cytotoxicity test as previously described[10] following the injection i.p. or i.v. of 0.05–5.0

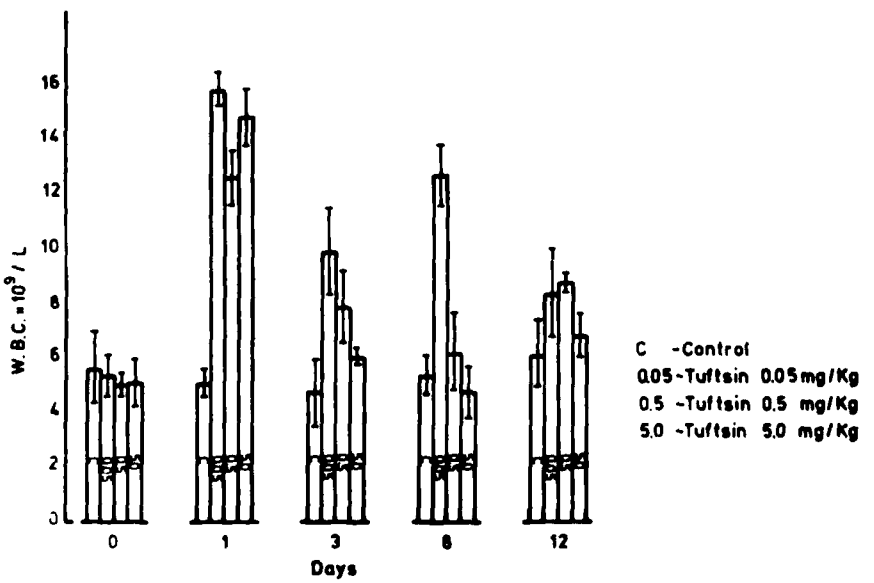

FIGURE 2. White blood cell counts in healthy BALB/c mice, 2–3 months old, following the intravenous administration of tuftsin.

mg/kg of tuftsin. The target cells were a ^{51}Cr-labeled RLδ1 cell line that originated from a radiation-induced T-cell lymphoma in BALB/c mice (kindly supplied by Dr. R. Herberman, The National Cancer Institute, Bethesda, Maryland). The results indicated that tuftsin induced a significant enhancement of the cytotoxic activity of spleen cells against the tested target cells. Only doses of 50 or 500 μg/kg caused such a significant increase. A dose of 5 mg/kg was ineffective. The effect of 50 μg/kg was most significant when tested seven days after injection (FIG. 3).

ANTITUMOR ACTIVITY OF TUFTSIN IN A MURINE FIBROSARCOMA MODEL

C3H/eb mice, eight to twelve weeks old, were used in these experiments. A transplantable fibrosarcoma, originally induced by 2-methyl-cholanthrene and maintained by serial intramuscular passages in syngeneic C3H/eb mice[11] was used as the tumor model. Tumor cell suspensions were prepared by trypsin-versan digestion of minced tumor tissue. The tumor cells were suspended in phosphate-buffered saline

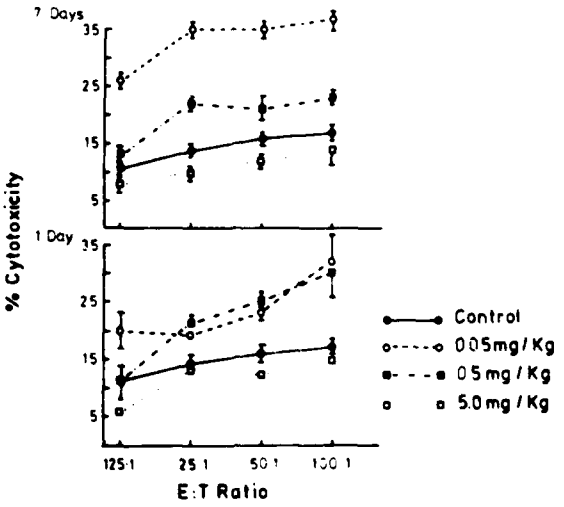

FIGURE 3. Effect of tuftsin on cytotoxic activity of spleen cells: C3H/eb mice were injected with different concentrations of tuftsin and their spleen cells were tested for cytotoxicity against RL δ 1 tumor cells.

(PBS) and a challenge of 1–3 $\times$ 10^4 tumor cells were given i.p. to the mice. Following tumor inoculation, a mass became palpable within 2–4 weeks, and the mice died within 30–50 days.

Tuftsin solution in PBS was administered i.p. in 0.1–0.2 ml at different time intervals after tumor inoculation. In some experiments, carrageenan (Sigma Chemicals Co., St. Louis, Mo.) was administered i.p. to mice in a dose of 0.5 mg per mouse in 0.2 ml of PBS.

Evaluation of the antitumor effect was performed in groups of 6–8 mice injected with tumor cells i.p. The mice were treated with either PBS alone or with tuftsin i.p. given three times per week for a total of three weeks starting on the day of tumor inoculation. Tuftsin was given in doses ranging between five μg/kg and 25 mg/kg. In one of the experiments, carrageenan was injected after each tuftsin administration. Survival was analyzed using the general Wilcoxon (Breslow) method.

There was a delay in the appearance of tumors in the tuftsin-treated mice, and a significant increase in their survival (FIG. 4). The antitumor effect was observed with doses between 10 μg/kg and 25 mg/kg. The median survival in the control mice was 21

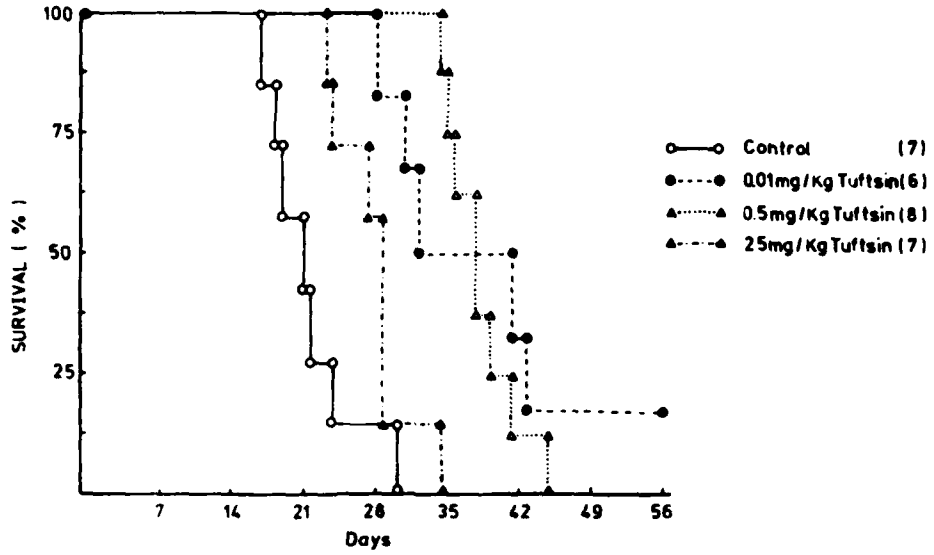

FIGURE 4. Cumulative proportion surviving curve, showing effect of i.p. tuftsin on the survival of C3H mice that received 2.0×10^4 fibrosarcoma cells i.p. on day 0. Tuftsin 0.2, 10, or 500 micrograms, was given three times a week for three weeks. The survival differences between the three tutftsin-treated groups and the control group are significant at the $p < 0.01$, $p < 0.001$, and $p < 0.05$ levels, respectively. (In parenthesis—number of mice in each group).

days, for those receiving 0.5 mg/kg it was 38 days, and in the group receiving 25 mg/kg it was 29 days. It is of interest to note that the highest dose of tuftsin was significantly ($p < 0.001$) less effective than the lower doses tested. The lowest dose used (5 μg/kg) had no effect on tumor growth and survival (data not shown).

Carrageenan, added concomittantly with each administration of tuftsin, 10 μg/kg,

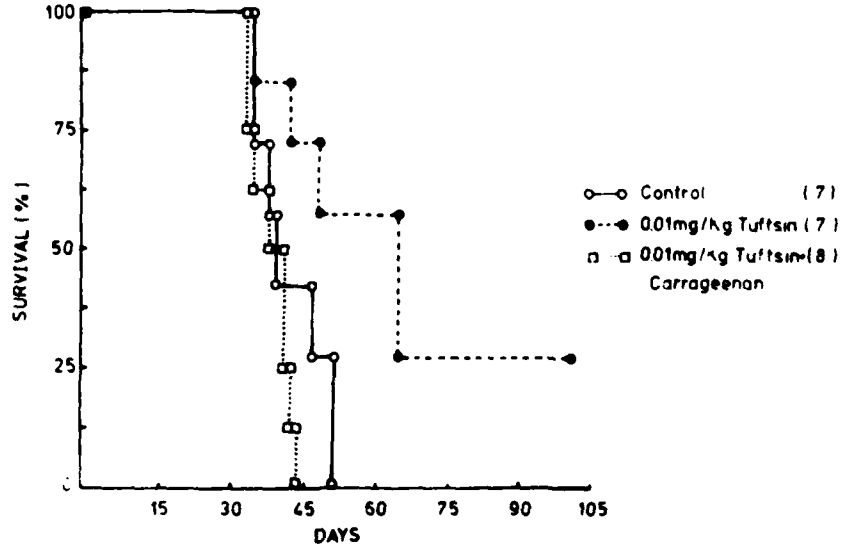

FIGURE 5. Cumulative proportion surviving curve showing effect of carrageenan, 0.5 mg/dose given i.p. after each dose of tuftsin three times weekly. This abolished the prolongation of life induced by tuftsin alone, 0.2 microgram/dose. C3H mice received 1.5×10^4 fibrosarcoma cells i.p. on day 0, and tuftsin was administered three times a week for three weeks.

completely abolished the antitumor effect of tuftsin (FIGS. 4, 5). Carrageenan alone did not have any effect on tumor appearance.

These results indicated that tuftsin has an antitumor effect in the murine fibrosarcoma tumor model used in the present study. The effective antitumor dose was significantly lower than the toxic dose (since the LD_{50} of tuftsin is more than 2 g/kg), and the optimal doses in the present set of experiments were between 10 μg/kg and 0.5 mg/kg. The reduction in tuftsin activity caused by carrageenan may suggest that the antitumor effect of tuftsin *in vivo* was at least partially mediated by activation of macrophages or other carrageenan-sensitive effector cells.

PRELIMINARY CLINICAL STUDIES

Fifteen adult patients with advanced cancer refractory to all conventional therapy were entered into a Phase-I study of i.v. administration of tuftsin. Their characteristics

TABLE 3. Characteristics of Patients in Phase-I Study of Tuftsin

Male/Female	9/6
Age-Median (Range)	58 (20–79)
Diagnosis	
Gastrointestinal tumors	7
Lung cancer	3
Sarcomata	2
Ovarian cancer	2
Nasopharyngeal carcinoma	1
Total (all solid tumors)	15

are shown in TABLE 3. Sterile tuftsin solution at a concentration of 50 mg/100 ml was prepared by ABIC Ltd., Ramat Gan, Israel. Treatment was started with a dose of 0.0025 mg/kg and the dose was escalated as shown in TABLE 4 up to 0.96 mg/kg. Tuftsin was injected slowly over a period of 1–3 minutes intravenously. Most of the patients received only one dose of tuftsin, but in a few cases repeated doses were given at two-week intervals.

In the first hour after injection, patients were monitored for pulse rate, blood pressure respiration rate, and temperature every 15 minutes and later every four hours. Blood counts were taken every day following tuftsin administration as well as SMA-C and blood samples for a lymphocyte cytotoxicity assay.

For testing lymphocyte cytotoxicity, 20 ml of heparinized venous blood were drawn and the mononuclear cells (PBM) were recovered by ficoll-hypaque centrifugation.[15] PBM, 5×10^5, were mixed with 10^4 [^{51}Cr]-labeled K562 cells in 0.2 ml of culture medium supplemented with 10% fetal bovine serum in U-shaped microtiter plates. The cells were incubated at 37°C. After an overnight incubation, the radioactivity in the supernatant was counted with a gamma counter and percent cytotoxicity was calculated as follows:

$$\frac{\text{CPM in experimental group} - \text{CPM in control}}{\text{CPM maximum release} - \text{CPM in control}} \times 100$$

The results of the various examinations and tests performed showed that tuftsin in doses up to 0.96 mg/kg had no acute or chronic toxic effects. The pulse, blood pressure, respiration rate, and temperature did not change following tuftsin administration. Similarly, all biochemical parameters remained unchanged. However, 7 out of the 15 patients receiving 0.02 mg/kg or more of tuftsin showed a >30% increase in WBC, and five patients had an increase of more than 50% in the WBC (TABLE 4). The leukocytosis was noted usually within 1–3 days and was maximal at 4–5 days. No significant change in the differential blood count was found following tuftsin administration.

The cytotoxicity of PBM against K-562 target cells was measured in eleven cases receiving tuftsin in doses between 0.08 mg/kg and 0.96 mg/kg. In eight of these cases, a significant increase (>100%) in the percent cytotoxicity was found (TABLE 4). The

TABLE 4. Effect of Tuftsin on WBC Counts and Lymphocyte Toxicity in Patients

Dose mg/kg	No. of Patients	No. of Patients with Increase >30% WBC	No. of Patients with Increase >50% WBC	No. of Patients with >100% Increase in Cytotoxicity
0.0025	2	0/2	0/2	ND[a]
0.005	1	0/1	0/1	ND
0.01	2	0/2	0/2	ND
0.02	2	1/2	1/2	ND
0.04	2	1/2	1/2	ND
0.08	3	0/2	0/2	1/1
0.16	3	1/2	1/2	1/2
0.24	3	1/2	1/2	3/3
0.48	3	1/3	0/3	3/3
0.96	2	2/2	1/2	0/2

[a]Not done.

cytotoxicity was generally found to be increased within the first week following tuftsin administration and decreased thereafter.

No objective tumor responses were recorded in the patients except for one patient who received a total of six injections of tuftsin and who had a significant neurological improvement, together with shrinkage of a nasopharyngeal tumor invading the base of the skull. This response lasted for two months and thereafter the tumor recurred and grew locally again.

The results of our Phase-I study in patients showed that a single administration of tuftsin given i.v. in doses up to 0.96 mg/kg is well tolerated. The only biological effects observed were an increase in the total number of peripheral blood leukocytes and in PBM cytotoxicity. The latter phenomenon seems to be dose dependent and most prominent with doses of 0.24 or 0.48 mg/kg tuftsin.

DISCUSSION AND CONCLUSIONS

This study presents a series of experiments that were designed to evaluate some of the biological effects of tuftsin in experimental animals and in patients with advanced cancer. Following its administration *in vivo*, tuftsin induced leukocytosis in mice, dogs, and humans, as well as enhanced cytotoxic activity of mononuclear cells in mice and humans, and showed significant antitumor activity in mice. All these activities were

mediated by doses of tuftsin ranging between 50 and 500 μg/kg body weight. Toxicology experiments performed in mice and dogs showed that doses of tuftsin up to 1 mg/kg body weight can be safely administered without any toxic effects. The acute lethal dose in mice was more than a thousand times larger, 2.4 g/kg. The most prominent biological effect of tuftsin in this dose range was a significant increase in the WBC in mice, dogs, and humans. In both dogs and humans, this increase was not accompanied by changes in the differential proportions of WBC. The mechanism of this phenomenon is as yet unclear. It may represent either a direct effect of tuftsin on WBC precursors, a margination phenomenon, or a stimulation of the bone marrow activity by factors secreted by activated macrophages.[13] The increase in WBC is a new aspect of tuftsin activity that may be of potential interest for some clinical problems, such as in the treatment of drug-induced leukopenia.

The other effects of tuftsin were its antitumor activity *in vivo* and its ability to augment mononuclear cell cytotoxicity *in vitro* in mice and humans. We have previously reported that in 60% of healthy individuals, tuftsin augmented the *in vitro* cytotoxicity of peripheral blood monocytes.[14] Similarly, the effect of carrageenan on the antitumor activity of tuftsin shown in the present study also indicates that this *in vivo* activity may be mediated by macrophages. However, there is still a possibility that other lymphoid cell populations are involved in tuftsin-mediated activity. One possible mechanism of action may be activation of macrophages resulting in the secretion of lymphokines that can cause leukocytosis and augmentation of lymhocyte cytotoxicity.[15,16] In previous studies, tuftsin was found to augment lymphocyte sensitization through activation of macrophages.[6] Additional studies are necessary to further elucidate the role of tuftsin in the regulation of leukocyte count and activity and its potential antitumor effect.

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The Antineoplastic Effects of Tuftsin and Tuftsinyltuftsin on B16/5B Melanoma and L1210 Cells[a]

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INTRODUCTION

The ability of tuftsin to stimulate killing of cells was demonstrated with several bacteria by Martinez *et al.*[1] They showed that within 15 minutes, tuftsin-stimulated mouse macrophages kill over 70% of *L. monocytogenes* at 20 mg/g of mice, whereas control macrophages show little or no killing at that time interval.

Florentin *et al.*[2] and Nishioka[3] showed that tumoricidal activity of macrophages from tuftsin-treated mice was much higher than in control animals. Further, the intraperitoneal injection of tuftsin into mice infected with L1210 cells resulted in a statistically significant prolongation of survival as compared to mice not receiving tuftsin injections. Similarly, there was a significant suppression of growth of injected syngeneic melanoma cells in tuftsin-treated animals.[3] A similar but dramatic effect of tuftsin was demonstrated in the suppression of growth of fibrosarcoma cells by Catane *et al.*[4,5] Najjar *et al.*[6,7] showed that with L1210 cells, tuftsinyltuftsin was more effective than tuftsin. In this report, we show that tuftsinyltuftsin is again more effective against B16 melanoma in syngeneic C57BL/6 mice.

MATERIALS AND METHODS

The following items were purchased: mice, DBA/2, from Jackson Laboratories (Bar Harbor, ME), C57BL/6 from Charles River Laboratory (Wilmington, MA), L1210 leukemia cells from American Type Culture Collection (Rockville, MD), and B16/5B melanoma cells from Charles River Laboratory.

Also purchased were: Dicyclohexylcarbodiimide (DCC), anisole, trifluoromethane sulfonic acid (TFMS) and Merrifield chloromethyl resin from Pierce Chemical Co. (Rockford, IL), and protected amino acids from Bachem Inc. (Torrance, CA).

All amino acids were protected as N^{α}-Boc. In addition, threonine was protected as the O-bz, lysine as N^{ϵ}-Z and arginine as N^{g}-tosyl derivatives. Coupling was done with DCC in dichloromethane.

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Tuftsin and tuftsinyltuftsin were synthesized by the solid-phase method of Merrifield[8] using TFMS[9] to cleave tuftsin from the resin as well as all suitably protected residues.[10]

RESULTS

In our laboratory, the effects obtained with tuftsin injections into DBA/2 mice that received L1210 cells, though marginally positive, like those of Nishioka's, did not represent an impressive tumoricidal effect. The accumulation of Lys-Pro-Arg through the action of aminopeptidase, given the high doses used, 20–25 μg per mouse, might inhibit tuftsin stimulation as shown *in vitro*.[11,12] Consequently, we synthesized the

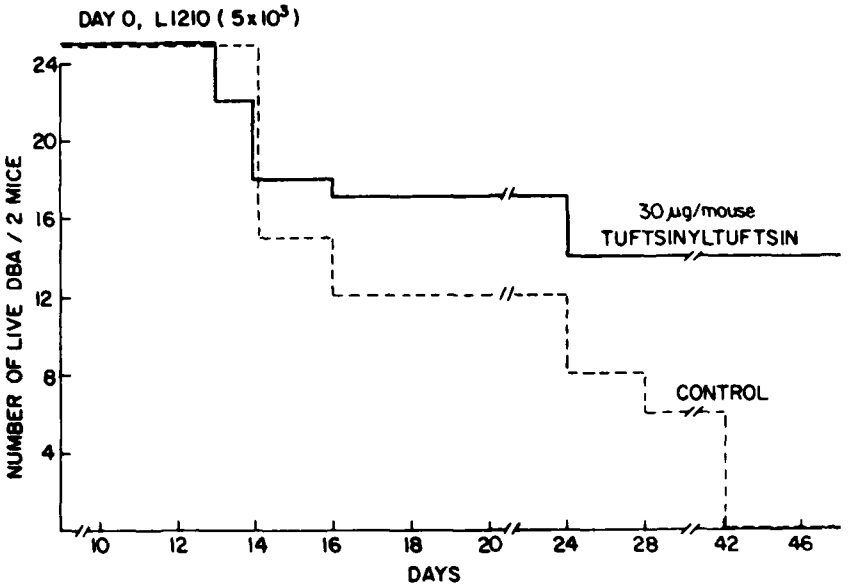

FIGURE 1. 25 DBA/2 female mice 11–13 weeks old were injected intraperitoneally (i.p.) each with 20 μg of tuftsinyltuftsin in phosphate-buffered saline (PBS), pH 7.5, on −7, −4, and 0 days at which time 5×10^3 L1210 cells were also injected i.p.

25 control mice received similar injections of PBS only. All animals were observed daily and dead mice scored.[6,7] $p = 0.001$.

dimer tuftsinyltuftsin with the expectation that this oligopeptide would yield tuftsin at a slow rate not sufficient to accumulate the inhibitory tripeptide. With that compound, we observed a dramatic effect that is depicted in FIGURE 1. We were also able to obtain a very significant prolongation of survival of treated mice over the controls, in addition to 35–40% survival over 60 days, at which point the animals were sacrificed. In each case, pancreas and lung tissue sections showed heavy infiltration in control animals and none in tuftsin-treated survivors (FIG. 2).

Our next attempt was with B16/5B melanoma cells 5×10^5 injected in C57BL/6 mice. Here the monomer tuftsin gave an excellent effect with 20 μg and 30 μg per mouse intraperitoneally (i.p.) at −7, −4, and 0 days followed by injections twice weekly. There was a considerable number of survivors, 38% of which showed no detectable tumors when sacrificed at 60–80 days. This is shown in FIGURE 3, which

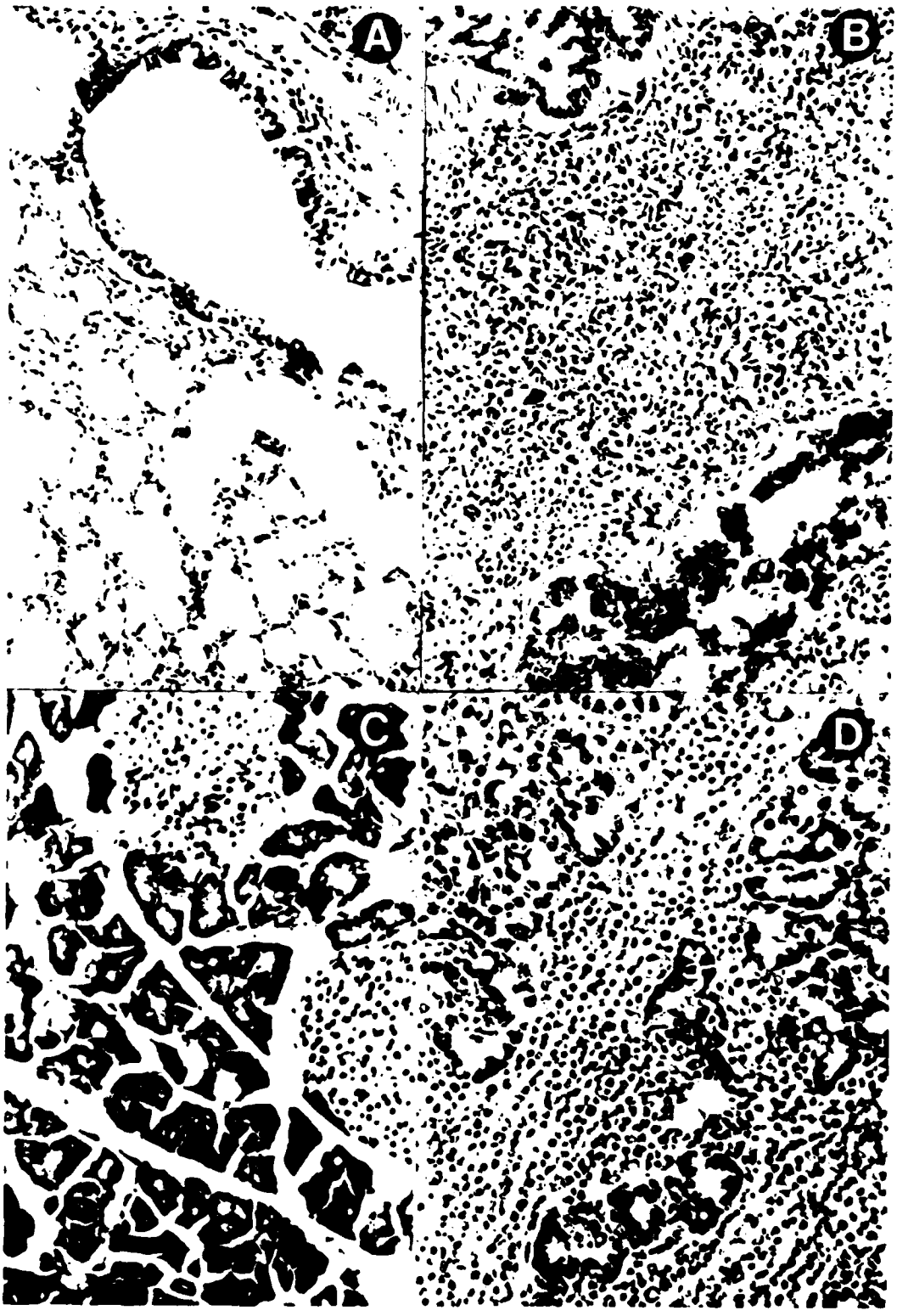

FIGURE 2. (A) Lungs of tuftsin-treated mice show normal histologic appearance. (B) Lungs of untreated control mice in which the architecture is obliterated by massive infiltration with L1210 cells. (C) Pancreas of surviving treated mice show normal acinar and islet tissue. (D) Pancreas of untreated control mice in which the interstitium of the acinar tissue is heavily infiltrated by L1210 cells.

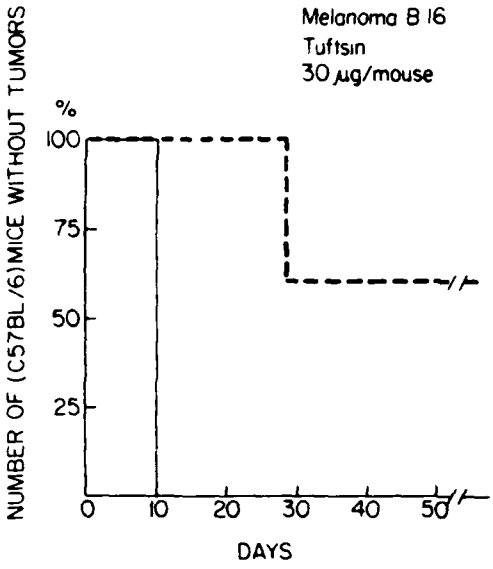

FIGURE 3. 25 C57BL/6 mice 12–14 weeks old were injected i.p. each with 20 µg of tuftsin in 100 µl of PBS on −7, −4, and 0 days at which time 5×10^5 B16/5B cells were injected subcutaneously (s.c.) in the right lower quadrant of the abdominal wall. This was followed by similar injections twice weekly. 25 controls were injected with PBS only. Animals were observed daily and the definite appearance of a subcutaneous tumor nodule recorded.[6,7]

Solid line represents controls and dashed line represents tuftsin-treated mice. $p < 0.001$.

illustrates several experiments. It is clear that the effect of tuftsin against B16 melanoma was very much more pronounced than against L1210 cells. The reason for this is not clear.

Encouraged by results obtained with 0.2 µg/kg body weight by Catane *et al.*,[4,5] we treated C57BL/6 mice i.p. with 3 ng per mouse three times at −7, −4, and 0 days, at which point they were injected subcutaneously with B16/5B melanoma cells 1.5×10^5. Tuftsin treatment was continued twice weekly thereafter. This is illustrated in FIGURE 4. The incidence of melanoma growth was significantly less than the control for the duration of the experiment. In two simultaneous experiments, with 2 µg and 20 µg per mouse, the tuftsin dimer yielded statistically similar results (FIG. 5). Obviously, we had been using too large a dose such that we could not obtain a dose-response effect.

Thus, several strains of mouse melanoma in C57BL/6 mice, L1210 leukemia cells in DBA/2 mice, 3-methylcholanthrene-induced, transplantable fibrosarcoma in C3H

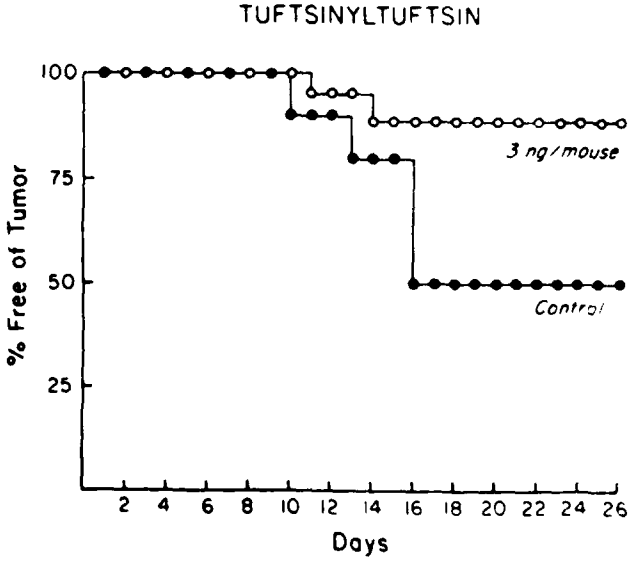

FIGURE 4. All details are as in FIGURE 3 except that the number of mice in each group was 16, 5×10^4 melanoma cells were injected, and 3 ng of the dimer tuftsinyltufsin was used instead of the monomer tuftsin.[6,7] Open circles represent treated mice and filled circles represent controls. $p = 0.01$.

mice, and murine virus sarcoma (type I) have responded very favorably to treatment with tuftsin or tuftsinyltuftsin.[3,4,6,7,13,14] The manner by which macrophages destroy tumor cells is under study by several groups and the lethal effects of H_2O_2, $O\cdot_2^-$, and $OH\cdot$ have been considered.[20,21] In this connection, it was shown by Tristch and Niswander[22] that tuftsin-activated macrophages produce considerable amounts of the potentially lethal superoxide anion, $O\cdot_2^-$ which has been shown earlier to result from the activity of NADPH oxidase.[23]

$$2O_2 + NADPH \rightarrow 2O\cdot_2^- + NADP + H^+$$

There are two other possibly lethal compounds that can be formed from the superoxide anion:

(1) Through superoxide dismutase hydrogen peroxide could be formed.[20]

$$2O\cdot_2^- + 2H^+ \rightarrow O_2 + H_2O_2$$

The hydrogen peroxide produced could enter the peroxidase-H_2O_2-halide system which has been shown to be lethal to tumor cells by several groups (see ref. 21).

$$X^- + H_2O_2 \rightarrow XO^- + H_2O$$

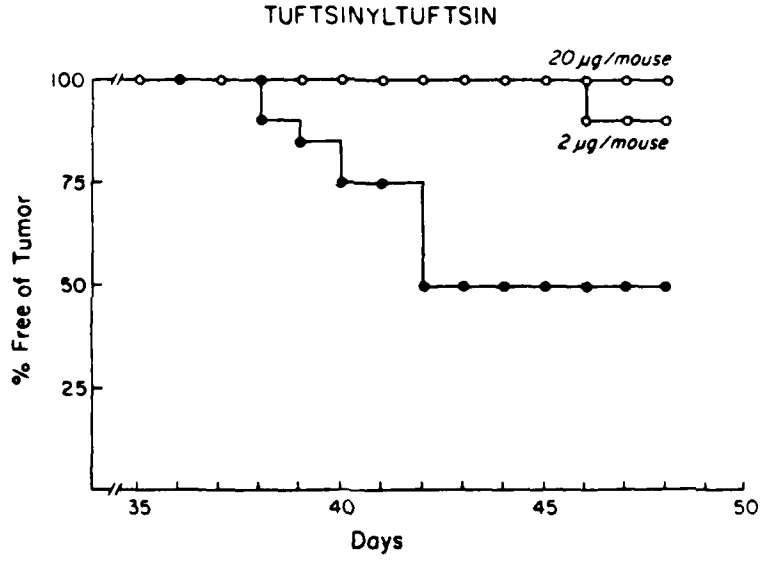

FIGURE 5. This figure represents two simultaneous experimental groups, 20 mice each, with a common control of 20 mice. The two experimental groups were injected with 2 µg or 20 µg per mouse. (see FIGURES 3 and 4 for details). $p = 0.07$ and $p = 0.02$.

The hypohalite XO^- can halogenate a molecule either to destroy or generate its function. This has been thoroughly reviewed by Morrison and Schonbaum.[24]

(2) The other pathway for superoxide is the generation of the $OH\cdot$, radical, known as the Haber-Weiss reaction.

$$O\cdot_2^- + H_2O_2 \rightarrow O_2 + OH^- + \underline{OH\cdot}$$

Another pathway for a haloginating system has recently been emphasized.[21] It is

suggested that iodination can be initiated by OH· as follows:

$$OH\cdot + I^- \rightarrow OH^- + I\cdot$$

The hydroxyl radical has been shown to be generated through the xanthene oxidase system.[21]

Thus the stimulation of $O\cdot_2^-$ generated by the phagocytic cells induced by tuftsin could augment equally well the levels of H_2O_2 and OH·. These potentially lethal molecules $O\cdot_2^-$, H_2O_2, and OH· may be responsible for the cytocidal activity of tuftsin-stimulated macrophages.

SUMMARY

The intraperitoneal (i.p.) injection of tuftsin, Thr-Lys-Pro-Arg, into C57BL/6 mice that were injected with B16/5B melanoma cells, resulted in a considerable suppression and elimination of solid tumor growth. While 100% of control animals exhibited tumor growth, 38% of the treated animals failed to show tumor formation for the duration of the experiment, 60–80 days. The octapeptide, tuftsinyltuftsin, was effective at 3 ng per mouse as was a dose of 2 and 20 μg per mouse. In each case there was a significant number of mice free of tumors.

The octapeptide was also quite effective against L1210 cells resulting in the survival of 35–40% of the treated animals.

The lethal effect of increasd superoxide, $O\cdot_2^-$, production by tuftsin treatmen may explain the antineoplastic effect of the tetrapeptide. This may result not only fron higher concentrations of $O\cdot_2^-$ but also from the potentially lethal effects of H_2O_2 anc OH· radical, both of which are products of $O\cdot_2^-$ metabolism.

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Potentiation of Antitumor Activity of α-Difluoromethyl Ornithine by the Immunomodulating Peptide Tuftsin

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INTRODUCTION

The naturally occurring polyamines (putrescine, spermidine, and spermine) have been shown to play an important role in cell proliferation.[1,2] α-Difluoromethyl ornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase,[3] the first enzyme in the polyamine biosynthetic pathway, causes a rapid depletion of intracellular levels of putrescine and spermidine and subsequent growth retardation in a number of cells grown in culture.[4,5] The intracellular depletion of polyamine levels by DFMO also results in inhibition of growth and metastases of tumors in experimental animals.[6-9]

Tuftsin is a physiologic tetrapeptide with immunoadjuvant activities.[10-12] Recently, tuftsin was also shown to possess immunologically mediated antitumor activity.[13,14] Tuftsin has been shown to potentiate macrophage and natural killer cell-mediated cytotoxicity against tumor cells.[15,16] We have found that administration of DFMO to mice bearing B16 melanoma results in a dramatic inhibition (80–85%) of tumor growth.[17] In the present study, we have investigated the usefulness of an immunomodulator such as tuftsin in potentiating the antitumor activity of DFMO in B16 melanoma in mice.

MATERIALS AND METHODS

Animals

C57 BL/6J mice (18–20 g) from Charles River were used for transplantation of the tumor. The animals were ' ~used in stainless steel cages with free access to food and water.

Drugs

D,L-α-Difluoromethyl ornithine (DFMO) was synthesized in our laboratories.[3] Tuftsin was synthesized[12] and analyzed for purity by high-performance liquid chroma-

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tography and amino-acid composition as described earlier.[18] DFMO was administered through the drinking water as a 2% aqueous solution starting 24 hr after tumor inoculation. The mean daily intake of the drug was found to be about 3 g/kg body weight. Tuftsin was dissolved in saline and administered at 25 μg/animal starting on day 2 and alternate days thereafter up to the 20th day (10 doses).

Tumor

B16 melanoma (F1) was kindly supplied by Dr. I. J. Fidler. The tumor was propagated and maintained *in vivo* by serial transfers of dissociated tumor cells in C57 BL/6J mice. To evaluate the effects of DFMO and tuftsin, an inoculum of 1×10^5 viable, dissociated tumor cells obtained as described earlier[17] were injected s.c into the interscapular region of the animals. Mice bearing tumors were sacrificed on the 21st day, the tumors were excised, weighed and a portion of the tissue was frozen at −70°C for polyamine analysis.

Polyamine Analysis

The tumor tissues were homogenized in 0.4 *M* perchloric acid and the supernatants obtained after centrifugation were used for polyamine determination by dansylation and subsequent thin-layer chromatography.[19]

ASSAY FOR MACROPHAGE-MEDIATED CYTOSTATIC ACTIVITY *IN VITRO*

Peritoneal macrophages obtained from untreated or drug-treated mice were plated into 35-mm dishes (10^5 cells/dish) in modified Eagle's medium. After 60 min of incubation at 37°, the monolayers were washed thoroughly to remove nonadherent cells, and B16 melanoma target cells were added to each dish (10^4/dish). The dishes were incubated at 37°C in a CO_2 incubator for 5 days. The cells were collected by brief trypsinization (1 min) and counted using a Coulter Counter. The percentage of cytotoxicity was determined as follows:

$$\% \text{ cytostatic activity: } 1 - \frac{\text{Cell no. remaining in experimental dish}}{\text{Cell no. remaining in the medium control dish}} \times 100$$

The values presented are the mean from 3 independent animals.

Student's t-test and Newman-Keuls multiple comparisons were employed for statistical analysis of the data.

RESULTS

Treatment of tumor-bearing mice with 2% DFMO in the drinking water resulted in 80% inhibition of tumor growth when compared to controls (TABLE 1). Tuftsin (25 μg/animal) given either i.p. or s.c. on alternate days for 10 doses caused only 23–26% inhibition of tumor growth, whereas a combination of DFMO and tuftsin resulted in

TABLE 1. Effect of Combination of DFMO and Tuftsin on the Growth of B16 Melanoma in Mice[a]

Treatment	Dose	Route of Administration	Tumor Weight (g; Mean ± SE, n = 10)	% Inhibition
Control			7.43 ± 0.50	
DFMO	2% in drinking water	p.o.	1.49 ± 0.22[b]	80
Tuftsin	25 μg/mouse	i.p.	5.70 ± 0.52[c]	23
Tuftsin	25 μg/mouse	s.c.	5.47 ± 1.44[c]	26
DFMO + Tuftsin	2% in drinking water + 25 μg/animal	p.o. + i.p.	0.73 ± 0.13[b]	90
DFMO + Tuftsin	2% in drinking water + 25 μg/animal	p.o. + s.c.	0.31 ± 0.20[b]	96

[a]1×10^5 B16 melanoma cells were injected subcutaneously in the interscapular region of C57/BL mice. The animals were divided into 4 groups of 10 animals each. The first group was on normal drinking water and served as the control. The second group received tuftsin 25 μg/animal either s.c. or i.p. starting on the 2nd day and alternate days thereafter up to the 20th day (10 doses). The third group received DFMO as a 2% aqueous solution in the drinking water (an oral dose of approximately 3 g/kg/day based on fluid intake) starting 24 hr after tumor inoculation until the end of the experiment. The last group of animals received a combination of both DFMO and tuftsin treatments as described above. At the end of 3 weeks, the animals were sacrificed, tumors were dissected and weighed. A portion of the tumor tissue was used for polyamine analysis. The results presented are the average of two experiments. Statistical analysis was done according to Student's t-test.

[b]Significant at $p < 0.005$

[c]Not significant.

TABLE 2. Effect of DFMO and Tuftsin on the Polyamine Concentrations of B16 Melanoma in Mice[a]

Treatment	Polyamine Concentration (nmoles/g)		
	Putrescine	Spermidine	Spermine
Control	36.0 ± 4.9	871.9 ± 82.6	590.2 ± 22.3
DFMO	1.5 ± 0.21	61.4 ± 6.7	528.4 ± 38.8
Tuftsin	27.0 ± 10.4	754.9 ± 303	527.3 ± 99.2
DFMO + Tuftsin	3.9 ± 0.25	73.0 ± 6.46	544.2 ± 23.5

[a]The tumor tissues were collected from the animals described in TABLE 1. The tissue was homogenized in 0.4 *M* perchloric acid and the supernatant obtained after centrifugation was used for polyamine determination by dansylation as described in the methods.

90–96% inhibition of tumor growth depending upon route of administration (i.p. or s.c) of tuftsin. Maximum inhibition (96%) was observed when tuftsin was administered s.c, closer to the tumor site. Microscopic tumors weighing 300 mg were found in animals receiving both DFMO and tuftsin. Inhibition of tumor growth by DFMO was associated with a decrease in putrescine and spermidine concentrations in the tumor tissue (TABLE 2). Tuftsin had very little effect on the polyamine concentrations in the tumor tissue whether administered alone or in combination with DFMO.

We have also investigated the role of macrophage-mediated tumor cytolysis in the treated animals in order to ascertain whether the observed potentiation of antitumor activity of DFMO by tuftsin is mediated through the enhancement of cell-mediated immunity in the host. The preliminary results presented in TABLE 3 indicate that animals treated with DFMO alone or in combination with tuftsin had significantly more peritoneal macrophages than the controls or animals receiving tuftsin. Furthermore, a marginal increase (9–10%) in the macrophage-mediated cytostatic activity was observed in animals receiving DFMO or tuftsin when compared to control animals. However, macrophages obtained from animals receiving both DFMO and tuftsin showed a significant increase (25%) in cytostatic activity compared to control animals.

TABLE 3. Effect of DFMO and Tuftsin on the Macrophage-mediated Cytostatic Activity *in Vitro* against B16 Melanoma[a]

Treatment	Yield of Macrophages/Mouse ($\times 10^6$; Mean ± SE, n = 3)	% Cytostatic Activity (Mean ± SE, n = 3)
Control	1.45 ± 0.20	27.4 ± 1.2
Tuftsin	1.25 ± 0.30[b]	37.0 ± 4.1[b]
DFMO	2.25 ± 0.30[c]	36.4 ± 4.1[b]
DFMO + Tuftsin	2.93 ± 0.13[c]	52.3 ± 6.8[c]

[a]1×10^5 B16 melanoma cells were injected s.c. in the interscapular region of C57/B1 mice. The animals were divided into 4 groups of 10 animals each and the DFMO and tuftsin were administered as described in TABLE 1. At the end of 2 weeks, the animals were sacrificed and peritoneal macrophages were collected, counted, and *in vitro* macrophage-mediated cytostatic activity was determined as described in the methods. The mean cell number in the plates with target (B16 melanoma) cells only is 2.8×10^5 cells.

[b]Not significant.

[c]Significant at $p < 0.05$.

DISCUSSION

The results of this study indicate that the combination of an effective inhibitor of polyamine biosynthesis and an immunomodulator can dramatically suppress the B16 melanoma tumor developed in mice. Recently we have reported a striking tumor suppression by a combination of DFMO and interferon, a known immunomodulator.[17] In the present study, we have demonstrated an enhanced natural cell-mediated tumor cytostatic activity in animals treated with a combination of DFMO and tuftsin. This augmented cell-mediated immunity could account for the observed tumor suppression in the animals. These results along with the relatively nontoxic nature of DFMO[6] indicate a potential use for the inhibitors of polyamine biosynthesis in combination with immunoadjuvants like tuftsin and interferon in cancer chemotherapy.

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